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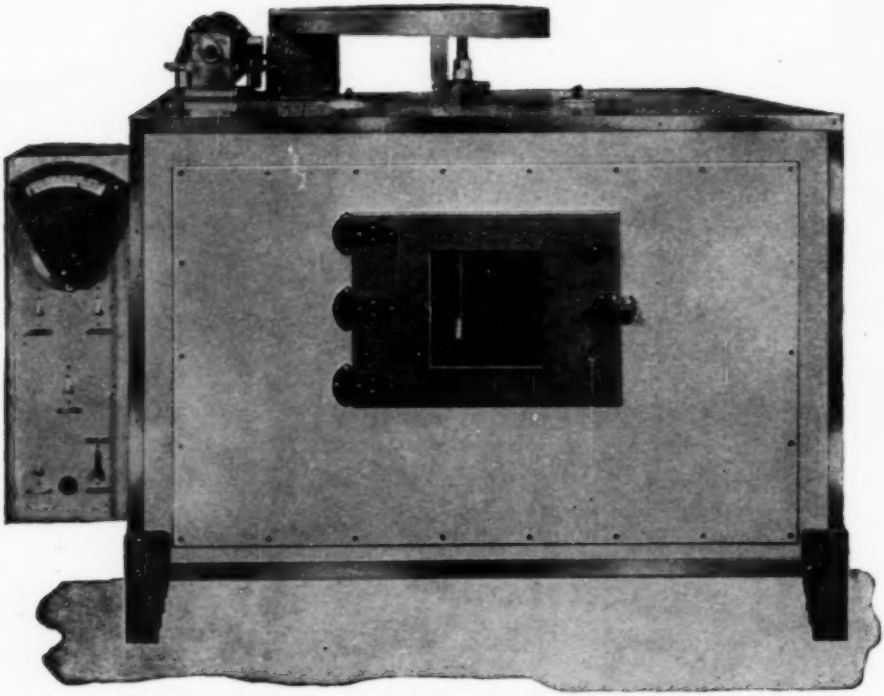

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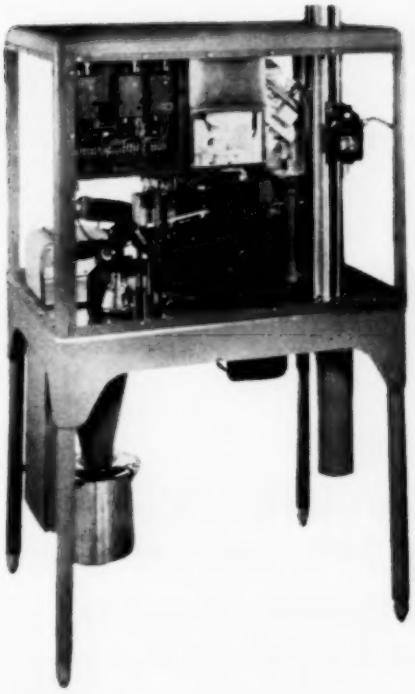

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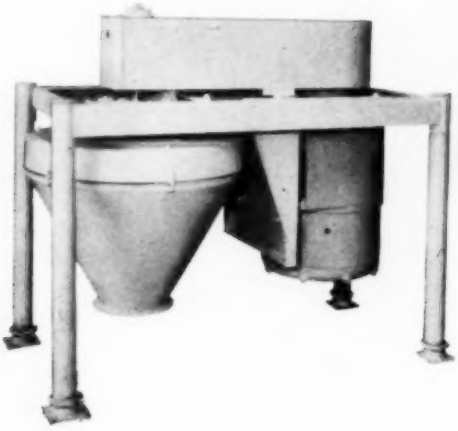

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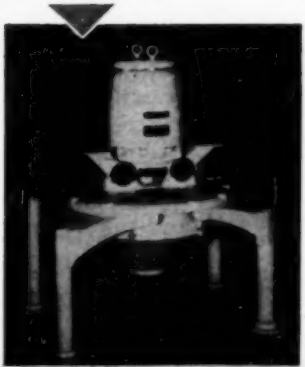

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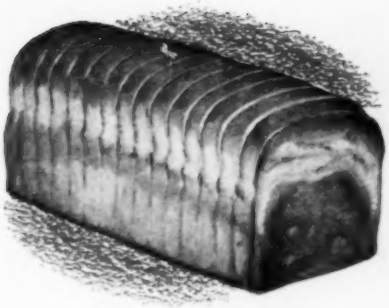

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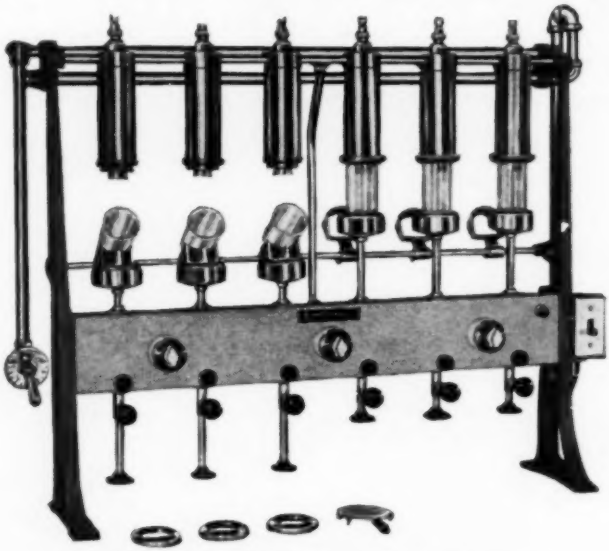

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CEREAL CHEMISTRY

VOL. 35 JANUARY, 1958 NO. 1

A REVISED THIOCHROME PROCEDURE FOR THE DETERMINATION OF THIAMINE IN CEREAL PRODUCTS[1]

W. G. BECHTEL[2] AND C. M. HOLLENBECK[3]

ABSTRACT

A procedure for the determination of thiamine in all cereal products is presented. This improves and simplifies the procedures given in the sixth edition of *Cereal Laboratory Methods*. In studies leading to the development of the new procedure it was found that drying the sample has no effect on the efficiency of extraction of thiamine, that sulfuric acid is a more convenient extracting agent than acetic acid, and that enzyme level and temperature can be selected to give a digestion of any desired time. A rapid method is given for activating Decalso. A Decalso column 3 cm. high is entirely adequate to adsorb thiamine. This permits discarding Decalso after a single use, thus eliminating its purification and reactivation. Results from three series of collaborative tests of a wide variety of cereal products show that the new procedure gives results at least equal in precision to those of other methods.

The basic steps for the thiochrome procedure for the determination of thiamine were reported by Jansen (15) in 1936. Following this, a number of investigations were made to compare results of this procedure with those of biological assay methods and to examine critically the steps in the thiochrome procedure. These have been reviewed elsewhere (2, 6). Because of the importance of thiamine in the enrichment of cereal products there are many papers describing its determination by the thiochrome procedure in these products, including several on the effect of variations in the basic method (4, 5, 6, 13, 14, 18, 22, 23).

The major responsibilities of technical committees of the Association were described by Anderson and Barackman (3). Briefly stated, they are to "aim continuously and specifically at improving, replacing, and adding to, the various tests and determinations published in *Cereal Laboratory Methods*." A committee is not necessarily expected to invent new methods.

[1] Manuscript received December 24, 1956. The studies on which this paper is based were made by the AACC Committee on Vitamins and Minerals in Enriched Cereal Products during the years 1953 to 1956.

[2] American Institute of Baking, Chicago, Illinois. Committee Chairman 1956-57.

[3] Wisconsin Malting Company, Manitowoc, Wisconsin. Committee Chairman 1953-1956.

With these responsibilities and limitations in view, the Committee on Vitamins and Minerals in Enriched Cereal Products made a study of the thiochrome method for the determination of thiamine. The reliability of this method and its applicability to cereal products had been proved by the investigations referred to above. It was the primary aim of the committee to endeavor to improve and simplify, if possible, the procedures given in the current edition of *Cereal Laboratory Methods* (1). Laboratory studies were made for the purpose of defining the most desirable conditions for each step in the method.

Experimental Methods

Each step of the procedure was studied in detail by one of the committee members. Studies of sampling procedures for cereal products of representative types, and of measurement of fluorescence, were made by surveys of procedures in several laboratories. Laboratory studies of the other steps of the procedure were made by individual committee members. Standard procedures were used except for the step under study.

On the basis of the results of these investigations a new procedure was written. This was subjected to collaborative study during a two-year period.

Results and Discussion

Results of the surveys of sampling and measurement of fluorescence are embodied in the new procedure, which is given below. Laboratory studies of other steps in the procedure are given in some detail, since they form the basis for the changes in method which are recommended.

All steps in the thiochrome method were studied exhaustively in the period from 1936 to 1946. Some of these studies were devoted to the application of the method to a wide variety of cereal products. Because of this, the task of the committee was greatly simplified since it was not necessary to prove the validity of the general procedure. It seemed necessary only to determine the allowable variations in each step so that the simplest and most convenient procedure, consistent with a high degree of accuracy, could be selected.

1. *Extraction*

Effect of Drying Bread. Bread, without being dried, was disintegrated and suspended in 0.1*N* sulfuric acid in a Waring Blendor. An aliquot, to contain the desired amount of bread, was used. Portions of the same bread were dried at room temperature to moisture levels of 7.6%, 6.0%, and 3.7%. Samples used for analysis had the same dry weight. The data given below indicate that drying the sample has no significant effect on the efficiency of extraction of thiamine.

Sample moisture (%)	34.4	7.6	6.0	3.7
Thiamine, mg/lb	1.19	1.22	1.21	1.26

Extraction Acid. Extraction with hot acid dissolves the various forms of thiamine which may be present. The low pH protects thiamine from rapid destruction during the heating process. A comparison was made between 2% acetic acid and 0.1*N* sulfuric acid for two heating periods, using a boiling-water bath. A reflux condenser was employed for the acetic acid. After cooling, acetic acid samples were treated with 5 ml. of 1.5*N* sodium hydroxide to bring the pH to 4.4. Sulfuric acid samples were treated with 5 ml. of 2.5*M* sodium acetate, which gave pH 4.8. The acids were found to be equivalent for each time of heating. Sulfuric acid is to be preferred for ease and convenience since it does not require refluxing, and for the ease with which it can be buffered by sodium acetate, to pH 4.5–5.0 required for enzymatic digestion.

Acid	*Adjusted pH*	*Thiamine*	
		Heated 15 min.	*Heated 30 min.*
		mg/lb	*mg/lb*
Acetic	4.4	1.28	1.21
Sulfuric	4.8	1.30	1.22

Time of Extraction with Acid. It is well known that heat is destructive to thiamine (5, 6, 11, 21). In the experiments on bread leading to the results given below, all samples were extracted with 0.1*N* sulfuric acid. Where 100°C. was used, the samples were submerged in boiling water for the indicated time.

	Temperature				
	100°C.			*121°C.*[a]	
Time (minutes)	10	20	60	5	15
Thiamine (mg/lb)	1.21	1.22	1.15	1.09	1.05

[a] Autoclave at 15 lb.

These results and those in the preceding section indicate a definite relationship between severity of heat treatment and recovery of thiamine. For this reason the time of heating the sample with acid should not be prolonged.

Enzymatic Digestion. The necessity for digestion of certain cereal products such as bread and wheat germ with enzymes has been demonstrated by several investigators (5, 8, 9, 14). The functions of the enzyme are to digest starch and to release free thiamine from its phosphate esters (8, 12). The latter, when oxidized, are not soluble in isobutanol. A number of studies of enzymes to find those suitable for this purpose have been made and it is generally known that Taka-Diastase[4], Mylase-

[4] Parke-Davis Co., Detroit, Mich.

P[5], and Polidase-S[6] are suitable, when 0.1 g. is used. A study was made of these enzymes, and the relationship between the amount of the enzyme and time and temperature of digestion. Results for bread are given in the tables below. All samples were 5 g., but different samples were used in various parts of the study.

Digestion 16 hours at 37°C.

Enzyme per sample (g.)	0.05	0.2	0.36	0.5	0.72
	Thiamine, mg. /lb.				
Taka-Diastase	1.19	1.17	1.19	...	1.20
Mylase-P	1.17	1.16	...	1.11	...
Polidase-S	1.19	1.13	...	1.15	...

Enzyme, 0.01 g. Digestion temperature 37°C.

Digestion time, hours	0	1	3	5	7	24
	Thiamine, mg. /lb.					
Taka-Diastase	0.93	1.06	1.10	1.12	1.10	1.19
Polidase-S	...	1.16	...	1.19	...	1.22

Taka-Diastase, 0.1 g. Total digestion time 25 hours

Hours at 37°C.	0	2	4	6	9	16
Hours at 27°C.	25	23	21	19	16	9
Thiamine, mg/lb	1.50	1.49	1.50	1.52	1.53	1.56

Taka-Diastase. Digestion 3.5 hours at 37°C.

Enzyme, g.	0.0	0.005	0.020	0.050	0.100	0.400
Thiamine, mg/lb	0.93	1.07	1.11	1.14	1.22	1.19

At the usual levels of 0.05 g. to 0.40 g. of enzyme, there is no difference in their effectiveness. At the level of 0.01 g. enzyme, differences are shown at lower digestion times. There is little, if any, difference between results of tests with samples digested 25 hours at 27°C. and those digested for two-thirds of the total time at 37°C. For a 3.5 hour digestion at 37°C. it is evident that 0.1 g. enzyme is sufficient. From these data it appears that enzyme level and digestion temperature can be selected to give a digestion time which best fits the assay schedule of the laboratory.

Thiamine may be adsorbed by filter paper. McRoberts (17) found that Whatman No. 41H and S&S No. 597 were satisfactory for filtration at this point. He recommended testing filter papers for suitability before using them.

2. *Purification*

This step separates thiamine from the remainder of the sample. It is necessary when the solution is colored or when the blank is high, indicating the presence of interfering materials. It may generally be omitted in tests of enriched flour and bread (5,10).

[5] Wallerstein Laboratories, New York, N.Y.
[6] Schwartz Laboratories, New York, N.Y.

The effectiveness of Decalso[7] and its superiority over other adsorbants for thiamine was demonstrated by Cerecedo and Hennessy (7). Since Decalso is entirely satisfactory, only a few tests of other adsorbants were made. In these tests and in those for column height a standard thiamine solution, calculated to give a galvanometer reading of 26.1, was used. In the tables below, results are reported in terms of the galvanometer reading.

Decalso	*Amberlite*[8] *IRC — 50*	*Amberlite*[8] *XE — 89*
26.2	22.1	9.5

Effect of height of Decalso column (cm.)

2	4	5	6	8
26.4	26.2	24.7	23.9	22.0

The amount of thiamine recovered decreased with increase in the amount of Decalso. There appeared to be an optimum quantity from which complete recovery of thiamine could be obtained readily. This was represented by a column between 2 cm. and 4 cm. in height. A column 3 cm. high requires 0.5 g. Decalso. This is capable of adsorbing 20γ of thiamine.

The necessity of the purification step for certain products is shown by the results in Table I. They show that purification is not required by flour or white bread, but is necessary for cereal products which give a colored filtrate. This confirms the findings of other investigators.

TABLE I

INFLUENCE OF PURIFICATION OF EXTRACT WITH DECALSO

SAMPLE	THIAMINE		DIFFERENCE
	With Column	Without Column	
	mg/lb	*mg/lb*	%
Breakfast cereal No. 1	5.83	5.08	13
	6.00	5.45	9
	6.05	5.24	13
Breakfast cereal No. 2	1.92	1.62	16
	1.95	1.62	17
	2.30	1.78	22
Breakfast cereal No. 3	5.45	4.65	15
	6.01	5.25	13
	5.65	4.70	17
Macaroni	3.38	3.18	6
	4.87	4.69	4
	4.82	4.61	4
Bread	1.48	1.57	5
	1.66	1.62	2
Flour	3.02	2.85	6

[7] The Permutit Co., New York, N.Y.
[8] Rohm & Haas Co., Philadelphia, Pa.

3. *Oxidation*

No part of the analysis has received a greater amount of study than that of oxidizing thiamine to thiochrome. Studies of the effect of the amounts of alkali and ferricyanide, of the order of addition, and of the time of oxidation have been reported (4, 9, 14, 23). To a certain extent at least, the results appear to depend on the type of sample analyzed (2).

Studies on enriched flour made by the committee indicate that results are not altered significantly by varying the amount of 15% sodium hydroxide between 2 ml. and 5 ml. Committee studies of the effect of the method and order of adding the alkali and potassium ferricyanide agreed with the results of other investigations. There was greater fluorescence when alkali was added first and followed by ferricyanide than when the order was reversed. Addition of alkaline ferricyanide gave results equal to those obtained by adding alkali first. When samples were added to alkaline ferricyanide the results were very low. The procedure of adding the mixed reagents to the sample was adopted because of its greater convenience.

No significant difference was found between results when the interval between addition of alkaline ferricyanide and shaking with isobutanol was between 30 seconds and 3 minutes. When thiochrome was extracted with isobutanol immediately after the reagents and sample were mixed, the standard was completely oxidized and had the maximum fluorescence, but the sample fluorescence was low. When a 5-minute interval was allowed, the fluorescence of the sample remained at the maximum while that of the standard had decreased, giving an erroneously high value. The same interval should be used for both sample and standard.

In some cases the aqueous and isobutanol layers separate completely. If they do not, the emulsion can be cleared by centrifuging. Centrifugation for 1 minute at 1800 r.p.m. was generally sufficient to obtain a clear alcoholic layer.

4. *Measurement*

Studies of photofluorometers for the thiamine assay have been made by Loofbourow and Harris (16), while filters have been studied by McRoberts (18). It was not possible for this committee to make a critical study of instruments and filters. It is believed that manufacturer's recommendations regarding filters should be followed. In the absence of such recommendations the primary filter should have maximum transmittance at approximately 360mμ. Corning type 584 is satisfactory. Secondary filters should be used to absorb primary

ultraviolet radiation. A combination of Corning type 038 and type 428 is satisfactory. Either quinine sulfate solution or a glass standard may be used for adjusting the instrument.

Thiamine in isobutanol is rather stable, but it is destroyed rapidly by ultraviolet light. Readings should be made without undue delay and the sample should not be exposed to bright light. When the sample has been placed in the instrument the reading should be made rapidly to avoid destruction of thiamine.

5. *Collaborative Tests of the New Procedure*

Results of collaborative tests conducted by the committee are given in Tables II, III, and IV. In calculating the mean and coefficient of variation, values more than 2 standard deviations from the mean of all values were omitted.

To determine whether differences in enzymes or in activation of Decalso might be the cause of variations in results in Tables II and III, each collaborator was supplied with the same sample, enzyme, and activated Decalso. Results with and without the adsorption column are given in Table IV. While there was a slight improvement in results over those in Table III, they were comparable to those in Table II. Differences in results must therefore be ascribed, in general, to other causes.

A number of collaborative tests for thiamine in cereal products have been reported (13, 14, 19, 20, 22). In most of these the coefficient

TABLE II

RESULTS OF COLLABORATIVE TESTS (1954–1955) OF THIAMINE CONTENT OF CEREAL PRODUCTS BY THE NEW PROCEDURE

(Eight collaborators)

	THIAMINE CONTENT OF SAMPLES AS RECEIVED				
	Corn Meal	Flour	Macaroni	Breakfast Cereal	Bread
	mg/lb	*mg/lb*	*mg/lb*	*mg/lb*	*mg/lb*
	2.8	2.7	3.9	4.5	1.9
	2.8	2.5	4.0	5.1	1.8
	2.9	2.4	4.0	4.6	1.7
	3.1	2.4	3.8	4.8	1.5
	4.3[a]	2.7	4.2	5.0	1.6
	2.4	2.2	3.4	4.3	1.6
	3.4	2.5	3.9	4.9	1.8
	2.5	2.2	3.4	3.4[a]	1.7
Mean	2.84	2.45	3.83	4.74	1.70
Std. Dev.[b]	0.31	0.18	0.27	0.31	0.12
Coefficient of variation, %	10.9	7.3	7.0	6.5	7.1

[a] Values omitted from mean and Std. Dev.
[b] Standard deviation of a single determination.

TABLE III

RESULTS OF COLLABORATIVE TESTS (1955–1956) OF THIAMINE CONTENT OF CEREAL PRODUCTS BY THE NEW PROCEDURE

(Eleven collaborators)

	THIAMINE CONTENT OF SAMPLES AS RECEIVED				
	mg/lb	*mg/lb*	*mg/lb*	*mg/lb*	*mg/lb*
	Bread	Flour	Breakfast Cereal	Shorts	Flour
	1.84	2.16	6.78	5.20	2.36
	2.15	2.30	8.00	9.00	2.60
	2.40	2.10	6.70	7.40	2.30
	1.86	1.90	7.75	6.82	2.69
	1.57	1.63	6.57	5.43	1.89
	1.88	1.94	6.08	5.52	2.01
	1.05[a]	2.01	7.10	7.60	2.22
	1.94	2.00	6.96	6.78	2.34
	1.75	2.25	6.81	8.16	2.24
	1.65	1.93	6.26	6.69	2.21
Mean	1.89	2.02	6.90	6.86	2.29
Std. Dev.[b]	0.260	0.195	0.568	1.180	0.221
Coefficient of variation, %	13.8	9.7	8.2	17.2	9.7
	Corn Meal	Baked Goods	Baked Goods	Whole Wheat	Breakfast Cereal
	2.56	4.95	2.26	1.67	2.74
	2.74	5.00	2.40	2.20	3.30
	...	...	2.40	...	3.10
	2.78	4.89	2.25	1.98	3.23
	2.86	5.22	1.79	1.93	2.83
	2.85	...	2.09	2.10	2.30
	2.80	4.64	1.83	2.14	3.73
	2.54	4.74	2.34	1.94	3.04
	2.34	3.89	2.04	2.04	3.37
	2.66	4.62	1.78	1.50	2.39
	2.57	4.30	2.36	2.27	3.19
Mean	2.67	4.80	2.14	1.98	3.02
Std. Dev.[b]	0.159	0.140	0.235	0.224	0.406
Coefficient of variation, %	5.58	2.90	11.0	11.3	13.4

[a] Values omitted from mean and Std. Dev.
[b] Standard deviation of a single determination.

of variation has been calculated or can readily be derived from the data. When the numerical values of the coefficients of variation in different papers are compared, it is necessary to note that some are based on the standard error of the mean, others on the standard deviation of a single determination. It is difficult to compare the work of this Committee with that of other collaborators. Earlier tests generally involved flour only, flour and bread, or flour mill streams, and did not include other cereal products. Where direct comparison could be made, the agreement of results of collaborative tests of the new procedure was found superior or equal to that of the earlier tests.

TABLE IV

COLLABORATIVE TESTS OF ONE FLOUR USING THE SAME DECALSO AND ENZYME
(Eight Collaborators)

	Thiamine	
	Adsorption Column	No Column
	mg/lb	*mg/lb*
	2.20	2.30
	2.16	2.38
	2.61	2.67
	2.37	2.65
	2.44	2.38
	2.19	2.12
	2.21	2.30
	2.50	2.62
Mean	2.34	2.43
Std. Dev.[a]	0.16	0.18
Coefficient of variation, %	6.8	7.4

[a] Standard deviation of a single determination.

6. *The New Procedure*

The procedure which follows has been approved by the Technical Policy Committee of the Association. In place of the two procedures in *Cereal Laboratory Methods* (1), a single procedure has been written which includes an explanation of the conditions under which the purification step may be omitted. It thus serves as both a complete and a simplified method. A section on sampling various cereal products is included. Sulfuric acid extraction followed by sodium acetate buffer replaces acetic acid and sodium hydroxide. This gives greater convenience. More specific directions are given for filtration. An improved, rapid method of activating Decalso is included, with an improved method for preparing the adsorption column.

Apparatus: (1) Fluorometer. Any of the instruments now on the market is suitable. In all cases, the operating procedures, arrangement of filters, and accessory equipment should be as recommended by the manufacturer.

(2) Water bath or steam bath for a temperature of 100°C.

(3) Incubator or water bath for a temperature of 37°–40°C.

(4) Decalso adsorption tubes. The reservoir should be approximately 50 mm. long and 25 mm. in diameter, and the adsorption tube 5 to 6 mm. inside diameter and approximately 140 mm. long, ending in a capillary tube 10 mm. in length and of such bore that when charged the rate of flow will be not more than 1 ml. per minute. (Chromatographic tubes, Fisher Scientific Co., 717 Forbes St., Pittsburgh 19, Pa.)

(5) Glass-stoppered cylinders or centrifuge separatory funnels of 25 ml. capacity.

Reagents: (1) Sodium hydroxide solution, 15%. Dissolve 15 g. of sodium hydroxide in water, cool, and dilute to 100 ml.

(2) Potassium ferricyanide solution, 1%. Dissolve 1 g. of potassium ferricyanide in water and dilute to 100 ml. If contained in a stoppered brown bottle and stored in a cool, dark place, this reagent is stable.

(3) Sulfuric acid solution, 0.1*N*. Dilute 2.8 ml. of concentrated sulfuric acid to 1 liter with water.

(4) Sodium acetate solution, 2.5*M*. Dissolve 205 g. of anhydrous sodium acetate or 345 g. of sodium acetate trihydrate in water and dilute to 1 liter.

(5) Enzyme preparation. Prepare fresh daily using a 2% solution or suspension of the enzyme in 2.5*M* sodium acetate. Any one of the currently marketed preparations is suitable including Taka-Diastase, Clarase, Polidase-S, and Mylase-P.

(6) Isobutanol. This should give a blank reading of 1.5 galvanometer scale divisions or less. If necessary, distill the commercial product in all-glass apparatus or shake it with activated charcoal to remove fluorescent impurities.

(7) Potassium chloride solution, 25%. Dissolve 250 g. of potassium chloride in water and dilute to 1 liter.

(8) Activated Decalso. Use the 50- to 80-mesh product. Activate the Decalso in a Büchner funnel. Wash a 100-g. portion of Decalso in the funnel with a 3% solution of hot acetic acid. Allow the acid to remain in contact with the Decalso for 10–15 minutes. Drain off the acid by vacuum. Then wash it with approximately 250 ml. of hot 25% potassium chloride solution, allowing this to remain 10–15 minutes before draining by vacuum. Repeat the acid wash. Next wash it with several portions of hot water until the extract is free from chloride as shown by a silver nitrate test. Dry the Decalso at room temperature or in an oven below 100°C. and store it in a stoppered bottle. Purified Decalso is available from Fisher Scientific Co., Pittsburgh, Pa., as *Thiochrome Decalso*.

(9) Stock thiamine solution. Dry thiamine hydrochloride (U.S.P. Reference Standard) over phosphorus pentoxide in a desiccator for at least 24 hours. Dissolve 100 mg. in 25% ethanol and dilute to 1 liter with 25% ethanol. This solution is stable for several months if kept at refrigerated temperatures.

(10) Standard thiamine solution. Dilute 5 ml. stock thiamine solution (warmed to room temperature) to 100 ml. with water. Dilute 4 ml.

of this intermediate concentration to 100 ml. with 0.1*N* sulfuric acid (final concentration 0.2γ per ml.). Prepare fresh daily. See Note 7.

(11) Quinine sulfate solution. Dissolve 100 mg. of U.S.P. Quinine Sulfate in 0.1*N* sulfuric acid and dilute to 1 liter with the sulfuric acid solution. Dilute 3 ml. of this solution to 1 liter with 0.1*N* sulfuric acid solution. Store in a brown bottle.

I. *Sampling:* (1) *Wheat, Rice, and other Whole-Grain Products.* Grind the whole-grain products in a laboratory mill to pass a 20-mesh screen. Thoroughly mix the milled material by tumbling or other suitable means.

(2) *Bread.* Use a known weight of air-dried slices representing a whole loaf or a half or quarter loaf. Dry the slices in a warm place with air circulation and reduced light until the sample is in equilibrium with the moisture of the air. Weigh the sample to determine the moisture loss in air-drying. Grind the air-dried sample to about 20-mesh in a laboratory grinder. Determine the moisture content of the air-dried sample by the usual oven moisture method (1). Calculate the thiamine content on the "as-received" basis or on moisture-free basis by correcting for moisture.

(3) *Corn Grits, Corn Meal, Puffed Cereals, Farina, and Flour.* Mix a large sample (at least several ounces) by tumbling the material in a suitable container or in a mechanical mixer. Grind the large-particle materials to 20-mesh or finer with a suitable laboratory mill. Tumble the milled material well.

II. *Extraction:* Take a weight of sample containing about 20γ of thiamine or such that the final portion (5 ml.) to be oxidized will contain approximately 1γ of thiamine (see Note 1). Place the sample in a 100-ml. volumetric flask, add 50 ml. of 0.1*N* sulfuric acid, and heat in a boiling-water bath for 10 minutes. Cool the flask to 40°C. or lower, add 5 ml. of enzyme suspension, reagent 5. Incubate at least 4 hours at 37°–40°C. Cool and dilute to 100 ml. Mix the digested sample extract thoroughly and filter through Whatman No. 41H or S&S No. 597 filter paper, discarding the first few ml. of the filtrate (see Note 2). If purification is not required, proceed directly to step IV, oxidation, using an appropriate aliquot (see Note 1).

III. *Purification:* It is necessary to use this step only on highly colored extracts or extracts with interfering substances. To determine if interfering substances are present, test duplicate samples with and without this step.

Add an aliquot of clarified filtrate (see Note 1) to the adsorption

column while the Decalso is still wet from the washings (see Note 3). Wash the reservoir and column with three successive 5-ml. portions of hot water to ensure the proper distribution of the thiamine on the column.

Elute the adsorbed thiamine from the Decalso by filling the tube with boiling 25% potassium chloride solution. (See Note 1 for volume of eluate to be collected.) This volume will contain all of the thiamine. Collect the eluate in a 25-ml. glass-stoppered graduate to permit the volume to be measured accurately.

IV. *Oxidation:* Mix the eluate well by inverting three or four times and pipet 5 ml. into a 25-ml. glass-stoppered, centrifuge separatory funnel (thiochrome reaction vessel). Pipet a similar 5-ml. aliquot into a second glass-stoppered, centrifuge separatory funnel to be used for the blank. Number these separatory funnels 1 and 2. To the first add 3 ml. of alkaline ferricyanide solution (see Note 4). To the second vessel add 3 ml. of 15% sodium hydroxide solution. Mix these gently for about 30 seconds and add 15 ml. of isobutanol to each. Shake vigorously for 60 seconds. Centrifuge to separate the layers (see Note 5). Draw off aqueous layer, add 1 ml. of 95% ethanol to the isobutanol layer, and mix. Decant about 10 ml. of the isobutanol into the cuvet for reading the fluorescence of the thiochrome.

V. *Measurement:* Determine the fluorescence on about 10 ml. of the isobutanol solution in terms of galvanometer deflections, operating the fluorophotometer according to the manufacturer's directions for the instrument used (see Notes 6 and 7). Subtract the blank reading from the corresponding sample readings.

Calculations:

$$\text{Mg. thiamine/lb} = \frac{R_x - R_{xb}}{R_s - R_{sb}} \times \frac{V}{Z} \times \frac{E}{5} \times \frac{1}{S} \times 0.454$$

where R_x = fluorometer reading with unknown;
R_{xb} = blank fluorometer reading with unknown;
R_s = fluorometer reading with B_1 standard;
R_{sb} = blank fluorometer reading with B_1 standard;
Z = volume in ml. of extract passed through zeolite tube;
E = volume in ml. of eluate collected;
S = sample weight in grams; and
V = volume of extraction (100 ml.).

NOTES: (1) The weight of sample, the aliquot to be put through the adsorption tube, and the volume of eluate to be collected for samples of various potencies are suggested in the table below: (Letters in parentheses are explained under *Calculations*.)

For sample containing:	*Weigh out (S)*	*Pass through zeolite tube (Z)*	*Collect eluate (E)*
mg/lb	*g.*	*ml.*	*ml.*
0–0.5	4	20	15
0.6–1.7	3	20	15
1.8–3.0	2	20	15
3.1–6.0	1	20	15
6.1–10.0	1	10	15
10.1–20.0	1	5	15
Over 20	1	2	25

(2) If the filtrate is turbid or if filtration is extremely slow, use a small amount of filter-aid or centrifuge the suspension prior to the filtration. If filter papers other than those recommended are used, test for thiamine adsorption by comparing filtered and nonfiltered standard solutions.

(3) Place 0.5 g. of previously prepared Decalso on a plug of glass wool in the adsorption tube. This amount of Decalso will make a column about 3 cm. high. Most procedures call for a Decalso column 8 to 10 cm. high, but generally the lesser amount is sufficient. If care is exercised in the preparation of the column, the solution passes through very rapidly and little trouble is encountered. The smaller amount of Decalso offers the opportunity for discarding the used product without having to go through a purification or reactivation procedure in order to save it. Wash the Decalso in the column with boiling water just before use.

(4) Prepare the alkaline potassium ferricyanide fresh daily by diluting 3 ml. of 1% potassium ferricyanide to 100 ml. with 15% sodium hydroxide solution. If preferred, add 3 ml. of alkali and 1 drop potassium ferricyanide separately, obviating the daily preparation of the mixture.

(5) In general, centrifuging for 60 seconds at about 1800 r.p.m. is sufficient.

(6) Make this measurement as quickly as possible to minimize the destruction of the thiochrome by the inciting light. It is advisable to conduct the measurements in a room with subdued light.

(7) Standardize the fluorometer with either a quinine sulfate solution or a glass standard. Periodic checks with the quinine sulfate solution or the glass standard during the day are desirable to keep the instrument standardized. If the initial reading is not given, readjust. Calibrate the galvanometer in terms of the deflection with a standard thiamine solution. With each set of samples treat one or two samples of the standard thiamine solution exactly as the unknown. These are both a blank and a sample solution, as described above. Use the standard thiamine solution at the concentration of 1 γ of thiamine per 5 ml. of final aliquot. The difference between the total and the blank readings of the galvanometer corresponds then to 1 γ of thiamine.

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WHITE RINGS IN FROZEN BREAD[1]

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ABSTRACT

During storage of bread in the frozen state, a whitened or opaque ring 1/8 to 1/4 in. in width developed in the crumb just beneath the crust. Length of storage before initial occurrence and subsequent rate of inward growth of the ring were dependent on the storage temperature. At 15°F. (−9.5°C.) the rings began to appear as early as 2 weeks after the bread was placed in storage; at 10°F. (−12°C.) about 5 weeks were required; and at 0°F. (−18°C.) 10 weeks or more were necessary.

The whitening phenomenon is associated with a movement of moisture by sublimation and diffusion from the interior of the crumb to a region of lower moisture content just beneath the crust. The effect can be produced by lyophilization of frozen crumb, provided its moisture content is above about 37%.

X-ray diffraction patterns of affected crumb did not appear to differ from those of normal crumb of similar moisture content. Microscopic examination, however, showed numerous tiny voids or pockets to be associated with starch granules in the whitened regions. Few such voids were found in nonwhitened regions. The increase in light scattering that results from the large increase in surface area associated with the voids is the probable cause of the whitened appearance.

Recovery of a normal appearance of the crumb did not occur upon defrosting, exposure to high humidity, or by heating to 140°F. (60°C.) for a few minutes, but this could be accomplished by moistening the crumb with excess water and allowing it to dry.

A previous paper from this laboratory (10) mentioned the occurrence of narrow, whitish bands or rings in the crumb of certain samples of frozen bread about 1/8 to 3/8 in. beneath the crust. The rather opaque appearance of the rings is reminiscent of freezer burn or store burn sometimes encountered in frozen meats or poultry (3, 8, 12). Until recently, no other reference to this phenomenon in frozen bread had been found in the literature except a passing mention that indicated such rings had been observed (2). However, acknowledgments by persons experienced in the freezing of bread suggest that the phenomenon is known, although it does not appear to be common. A brief discussion of conditions causing and affecting this altered appearance of bread crumb was published recently (13).

The present paper summarizes results of experiments designed to determine conditions causing formation of the rings and to discover a mechanism by which they form. Variables investigated included formulas, rates of freezing, rates of defrosting, and storage of frozen

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breads at different temperatures for different times. Microscopical and X-ray diffraction examination were made of both normal and whitened crumb produced either by storage or by freeze-drying in the laboratory. A detailed examination of moisture distribution in certain samples of frozen breads was also made.

Materials and Methods

The commercial bread used in these experiments was large-sized (22.5-oz.) white pan bread made by conventional sponge and dough methods, sliced, wrapped in waxed paper, and obtained from the bakery about 2 hours after baking. Straight-dough laboratory breads were made by the AACC procedure (1) and the formula of Finney and Barmore (4). Starch and wheat protein fractions were prepared and incorporated into doughs according to methods previously described (9). Emulsifying agents were dispersed in aliquots of the vegetable shortening used for the laboratory breads before being added to the straight doughs. Both polyoxyethylene stearate (Myrj 45, R. T. Vanderbilt Co., 33 Winfield St., East Norwalk, Conn.) and monoglyceride (Myverol 18-40, Distillation Products Industries, 630 5th Ave., New York 20, N.Y.)[1] emulsifying agents were used at levels of 10 and 13% of the shortening.

Bread samples were freeze-dried in a desiccator attached to a mechanical high-vacuum pump through a dry ice-acetone moisture trap. Moisture contents were determined by the AACC vacuum-oven method (1). X-ray diffraction patterns were obtained under vacuum, using nickel-filtered copper radiation on crumb samples sealed in glass capillary tubes to prevent loss of moisture (7). Different moisture levels of the bread samples for X-ray examination were obtained in some instances by equilibration of crumb samples over saturated salt solutions of the type and concentration necessary to provide atmospheres of suitable relative humidity. Frozen bread samples were stored in cold rooms at temperatures automatically maintained within about ±2°F. (1°C.).

Individual experiments utilizing these methods and materials are described in detail at appropriate places in the following section in connection with presentation and discussion of the results.

Results and Discussion

Description of Visible Effects. The whitened appearance of certain portions of the crumb of frozen bread was first observed in commercial bread that had been stored for 6 or more months at 0°F.

[1] Mention of commercial products does not constitute endorsement by the Department of Agriculture over others not mentioned.

(−18°C.). Later it was found to occur much more quickly, especially at higher storage temperatures, and to be a progressive phenomenon. The first region of a loaf to be affected was most often the central area of the heel slice and the outer face of the slice adjacent to the heel. Almost as quickly, a ring of affected crumb began to form a short distance beneath the crust of all slices of a loaf. As storage continued, the affected areas began slowly to extend toward the center of the loaf both from the heel ends and from the periphery of the central slices. In some cases the whitening eventually extended completely to the center of the slices. Seldom, if ever, did the whitening extend outward completely to the crust. The whitened areas are clearly visible both when the bread is still frozen and after it has been defrosted. Normal frozen crumb is slightly whiter and more opaque than unfrozen crumb, and numerous glints of light reflected from ice crystals are seen, especially near the center of the slices. This milder whitening effect is reversible upon defrosting, but the more intense whitening effect that occurs after storage is incompletely reversible. The contrast between normal and affected areas can be eliminated by moistening defrosted whitened crumb with water and allowing it to dry, but exposure to a humid atmosphere for several days only partly restores the original appearance. Refreshening of the crumb by heating to 140°F. (60°C.), or above, for a few minutes does not reverse the phenomenon.

Time, Temperature, and Formula Effects. In a systematic study of conditions that might influence the formation of white rings in freshly baked commercial bread, rapid and slow freezing combined with rapid and slow defrosting were found neither to produce the rings nor to affect the time required for their occurrence during subsequent storage. Different loaves from a single large lot were frozen either in appoximately 75 minutes or in 6 to 7 hours, and portions of each of these sub-lots were defrosted over a period of either 3 to 5 hours or about 16 hours. In frozen bread stored at different temperatures, white rings were observed in about 2 weeks in loaves stored at 15°F. (−9.5°C.), irrespective of freezing treatment. At 10°F. (−12°C.), between 4 and 5 weeks were required before signs of whitening were visible. At 0°F. (−18°C.) nearly 11 weeks passed before definite signs of whitening appeared. Similar results were obtained with straight-dough breads baked in the laboratory.

Omission of shortening or milk solids from experimental laboratory bread had no effect on the time required for white rings to appear during storage at 15°F. (−9.5°C.). Addition of normal amounts of either monoglyceride or polyoxyethylene stearate emulsifying agents

likewise had no influence on the rate of formation of rings. Enrichment of doughs with extra gluten and soluble proteins, however, delayed appearance of rings for 3 or 4 days at the 15°F. (−9.5°C.) temperature. Dilution of the flour by addition of starch hastened the changes so that rings appeared in 10 or 11 days in loaves stored at 15°F. (−9.5°C).

Moisture Relationships. The similar appearance of whitened, more opaque than normal regions of bread crumb and the surfaces of meats that have been affected by freezer burn suggests a similarity in the mechanism of their formation. Freezer burn or store burn of meats is believed to result from surface dehydration by sublimation of ice from the tissues. The resulting numerous small voids or holes scatter more light so as to increase the opacity of the affected surfaces (8, 12). In meats, as in bread, the changes are essentially irreversible, although a partial reversion of the whitening occurs upon defrosting (3, 8). With meat an actual loss of moisture from affected surfaces occurs, but with bread no decrease in total moisture of a loaf appears to be necessary, although moisture contents of affected regions decrease substantially.

White rings occurred after the usual storage times at a given temperature in commercial bread that had been wrapped in aluminum

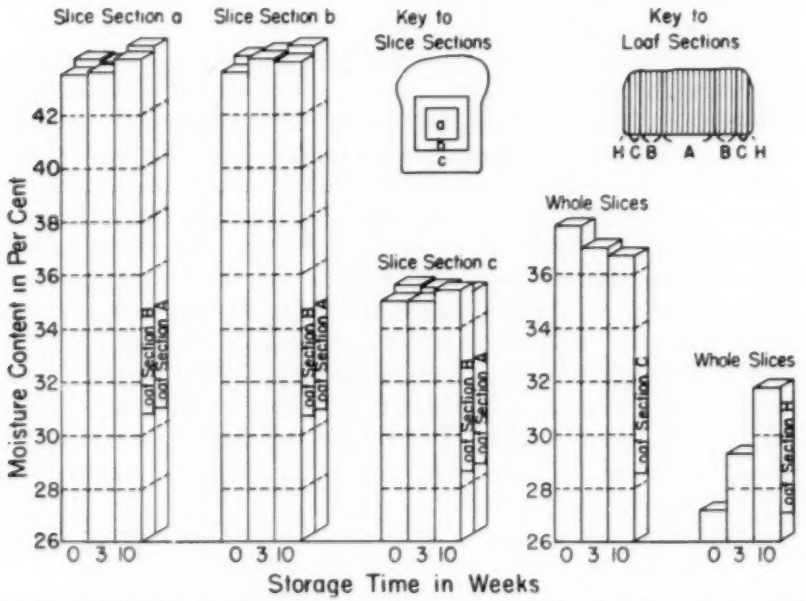

Fig. 1. Distribution of moisture in large sections of slices from several parts of loaves of frozen bread stored at 15°F. (−9.5°C.).

foil and sealed with cellulose tape so that no loss of total moisture from the loaf could occur, other than a small amount that collected as frost on the inner surface of the wrapper. This observation suggested that an internal movement of moisture during frozen storage might occur and be responsible for whitening, even though the total moisture and the gross distribution of moisture in a loaf of frozen bread had previously been found to be essentially constant for at least as long as 7 weeks (11).

Figure 1 summarizes measurements of distribution of moisture in the foil-wrapped frozen bread that had been stored for 10 weeks at 15°F. (−9.5°C.). In full agreement with previous results, there was no appreciable change in the distribution of moisture throughout the loaf. Only the two slices near each heel (see Fig. 1) lost a significant amount of moisture, and this seemed to have been taken up by the heels. These results show that loss of moisture from bread and a gross movement of moisture within loaves are not necessary for white rings to form. (After more than a year's storage at 0°F. (−18°C.) the white rings in other loaves of this lot of foil-wrapped bread had not increased in size. Very likely the inward growth of white rings in bread wrapped with waxed paper during long storage is due to a slow loss of total moisture through the wrapper.)

The sections of crumb in which moisture was determined in this experiment were rather large, however, and there seemed a good possibility that a rather steep moisture gradient existed in the crust sections, as cut. A movement of moisture by sublimation and diffusion could easily occur at slow rates within this section. Figure 2 shows the approximate position that white rings usually occupy, superim-

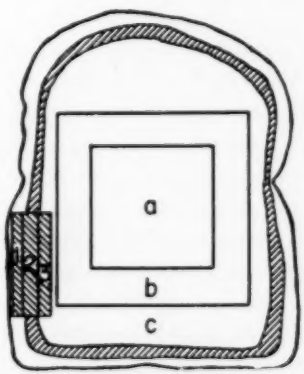

Fig. 2. Diagram indicating sections of slices from which moisture samples were obtained and the position normally occupied by white rings shortly after their first appearance.

posed on the pattern in which the slices were cut for determination of the gross moisture distribution. To check on moisture distribution and possible movement within the crust section (c), samples of crumb from locations indicated by the numerals in Fig. 2 were taken for moisture determination. The small samples were taken after different intervals of storage from some of the foil-wrapped bread used in the experiments summarized in Fig. 1. The samples were dissected from center slices of the loaves in the cold room in which they were stored in order to minimize any condensation of atmospheric moisture during the sampling. The bread stored at 15°F. (−9.5°C.) had begun to show signs of white rings after about 2 weeks of storage, but bread held at 0°F. (−18°C.) had no visible evidence of such rings after 10 weeks of storage.

The moisture values of bread stored at 15°F. (−9.5°C.) (Table I) indicate a progressive decline in moisture from zone 2 that appears to

TABLE I

MOISTURE CONTENTS, IN PERCENT, OF CRUMB REGIONS ADJACENT TO THE CRUST OF FROZEN BREAD STORED AT 15°F. (−9.5°C.) AND 0°F. (−18°C.)

(See Fig. 2 for key to slice sections)

ZONE No.	WEEKS OF STORAGE		
	4	7	10
	Stored at 15°F.		
1	31.7	32.8	33.2
2	37.7	36.9	36.8
3	43.7	43.2	43.3
	Stored at 0°F.		
1	42.3	39.4	39.8
2	44.1	44.2	43.6
3	43.8	43.8	43.7

be absorbed in zone 1. Zone 3 shows no significant decline in moisture. In bread stored at 0°F. (−18°C.) both zone 1 and zone 2 showed at least a slight loss of moisture in 4 to 7 weeks or 7 to 10 weeks, respectively; but zone 3 showed no change. Apparently, the loss of moisture from these crumb regions, that seems to accompany white-ring formation, had not progressed enough in bread stored at 0°F. (−18°C.) for the visible changes to have appeared.

Whitening by Lyophilization. When a slice of frozen bread containing a white ring is allowed to defrost and to dry at room temperature the ring remains clearly visible and the central portion of the slice retains its normal semitranslucent appearance. If a similar slice of bread is freeze-dried, the entire central portion of the slice inside the original white ring also becomes white. This is illustrated in Fig. 3

with bread to which orange food coloring was added to provide the necessary contrast for photographic reproduction of the affected regions.

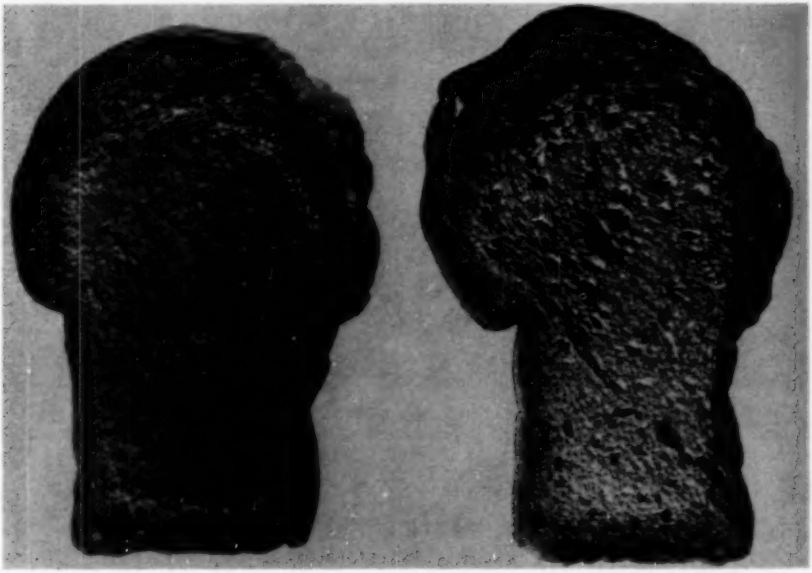

Fig. 3. Photographs of (left) slice from a "one hundred" loaf containing added coloring to show location of the white ring; (right) a similar slice after lyophilization to show whitening all the way to the center.

The fact that the whitening effect was confined to the more moist regions of the crumb again suggests that a minimum moisture content is required for its occurrence. In confirmation of this hypothesis, a slice from a freshly baked loaf showed whitening to within a very small distance from the crust after lyophilization (Fig. 4, left). When an unsliced loaf of the same bread stood at room temperature for 3 days without a wrapper, to permit a considerable loss of moisture, the whitened area in a lyophilized slice was confined to a region beginning more than an inch inward from the crust (Fig. 4, right). The regions of the slices outside the rings appear to be unaffected because of too low a moisture content.

To determine the minimum moisture content required for the whitening effect to occur upon freeze-drying, slices of bread crumb from which the crusts had been trimmed were suspended in air to dry slightly. After varying intervals, the crumb slices were sealed in small polyethylene bags and allowed to stand for 2 days to come to moisture equilibrium. The slices were then cut in two; one half was

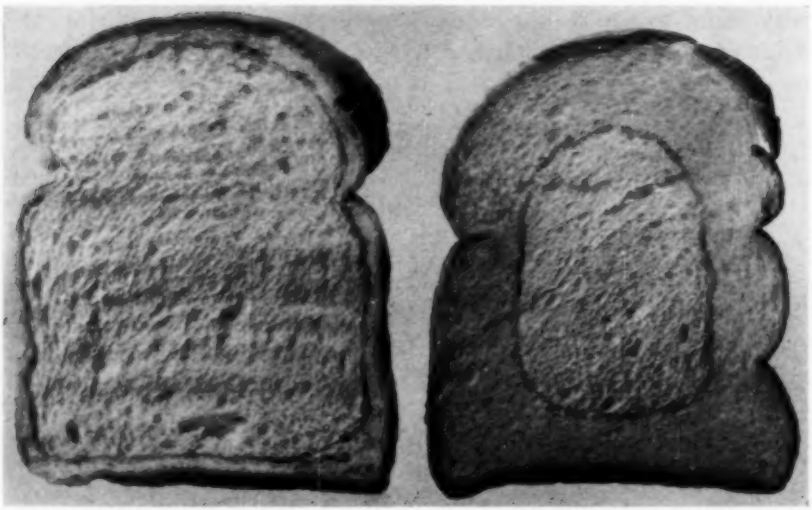

Fig. 4. Photographs of (left) slice of commercial bread frozen and lyophilized about 5 hours after baking; (right) slice of commercial bread frozen and lyophilized after unsliced loaf had stood 3 days without a wrapper. Margins of whitened areas were traced in pencil.

used for determination of total moisture while the other half was frozen and lyophilized. In this manner, an approximate minimum moisture content of 35 to 37% was found to be required for crumb whitening upon lyophilization of frozen bread.

X-Ray Diffraction Examination. Minute comparison of X-ray diffraction patterns of crumb from normal and whitened regions of the same slices, or of unfrozen control bread, showed no differences of significance in samples of comparable moisture contents. Diffraction patterns were obtained on samples of crumb from several depths beneath the crust of bread that had been stored in waxed wrappers (a) at 0°F. (−18°C.) for 2 weeks, (b) at 15°F. (−9.5°C.) for 8 and 11 weeks, (c) at 0°F. (−18°C.) for more than a year, and (d) without wrappers at 70°F. (21°C.) for several weeks under either the ambient or one of several controlled relative humidities.

Diffraction patterns in nonwhitened portions of crumb from either unfrozen or frozen stored bread were essentially alike and quite similar to the B patterns designated by Katz (5, 6), as long as the moisture contents of the crumb were approximately normal for inner portions of loaves (42 to 44%). This was true whether or not the bread was stored only 2 weeks or more than a year at 0°F. (−18°C.), or up to 11 weeks at 15°F. (−9.5°C.). In whitened portions of the bread stored at 15°F. (−9.5°C.), however, a definite decrease of pattern intensity

was found when observations were made after 8 weeks and again after 11 weeks of storage.[5] In bread stored for a year at 0°F. (−18°C.), the diffraction patterns of the whitened portions had faded sufficiently to indicate an almost complete loss of crystallinity of the starch.

The moisture content of the whitened crumb of the year-old bread was approximately 20%, whereas that of the bread 11 weeks old was about 29%. Similarly faded patterns were found in the unfrozen bread samples when crumb moistures at the time of sampling were of similar low magnitudes. Moreover, crumb samples taken from the clearly discernible white ring of the bread stored 11 weeks at 15°F. (−9.5°C.) and from either side of the ring showed a weakening of pattern intensity that was progressively greater toward the crust and was more closely correlated with the moisture gradient across the region than with the discrete visual differences. These findings indicate, therefore, that the changes in molecular organization shown by X-ray diffraction are primarily a function of moisture content and appear to have no direct relation to formation of white rings.

Microscopical Observations. Microscopical examinations of bread

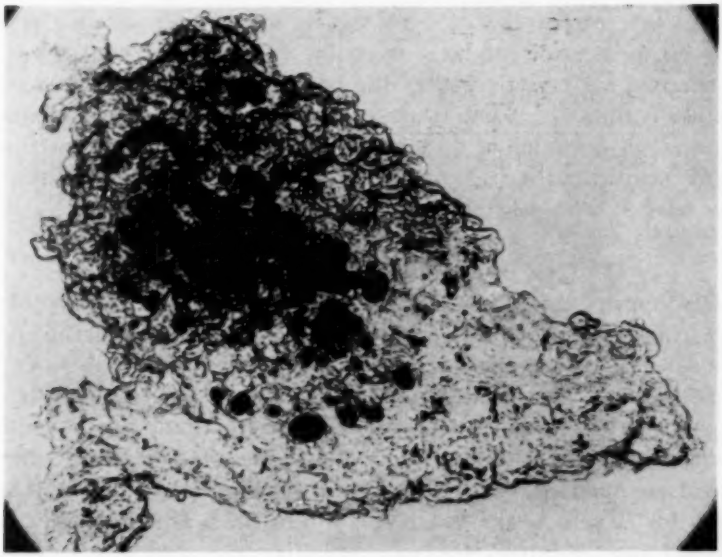

Fig. 5. Photomicrograph of a fragment of whitened bread crumb illustrating the association of cavities (black bubbles) with starch granules (magnification 120 diameters). The paraffin oil used for mounting has completely or partially filled many of the cavities. The largest bubble (at bottom of picture) almost completely fills the starch granule associated with it.

[5] Although whitening of portions of the crumb had begun much earlier, complete rings were only faintly visible at 8 weeks. At 11 weeks they were clear enough and wide enough to permit confident sampling of the desired regions.

crumb at 0°F. (−18°C.) and at room temperature by both reflected and transmitted light revealed the presence of numerous air-filled cavities in the whitened portions of the crumb. These cavities appear to be chiefly, if not exclusively, within the granules of starch scattered through the crumb structure (Fig. 5) and are responsible for the scattering of light that causes the unusual whitened appearance. Not all of the starch granules contain cavities. The thinnest septa between large gas bubbles seldom show such cavities. Normal crumb contains few, if any, similar cavities.

Each cavity appears to occupy part of the original volume of the gelatinized starch granule and could result from shrinkage of the gelled granule during dehydration. The proteinaceous matrix structures would have to be strong and rigid to avoid collapse of any void so formed. A more likely mechanism might be the growth of a crystal of ice within the gelatinized starch granule followed by sublimation of the ice to leave a cavity.

Observations made at 0°F. (−18°C.) indicate that no ice is present in whitened zones, but that much ice is present in adjacent inner zones which became whitened if lyophilized or simply allowed to dry in the open air of the cold room. So many prismatic ice crystals occur on surfaces of the large gas-pores of the crumb that direct observation of ice crystals within the boundaries of the swollen starch granules is difficult. Some evidence of the presence of ice within the granules was obtained with a polarizing microscope. Sublimation of this ice into initially drier zones of the loaf would leave cavities like those seen in whitened crumb.

General Discussion

The approximate time intervals required for occurrence of the white rings in frozen bread stored at 15°F. (−9.5°C.) and the incomplete reversal of the effect after defrosting, observed in these experiments, confirm the recently published observations of Walker (13). No particular requirement for temperature fluctuations as a required condition for formation of the rings was observed, however. In previous experiments (11) no white rings were noticed in frozen bread stored for seven weeks at temperatures fluctuating between 0°F. (−18°C.) and 20°F. (−7°C.) on a 24-hour cycle. The temperature at the center of these loaves varied only between 0°F. (−18°C.) and 14°F. (−10°C.), however, and any acceleration of the whitening effect may have been too small for changes in visible appearance to occur within the observation period. Nevertheless, even very small fluctuations in temperature should aggravate the internal movement of moisture and

would logically be expected to hasten the occurrence of the white rings.

The whitened appearance of affected crumb undoubtedly is due to the increased scatter of light caused by formation of the tiny voids or cavities found in such numbers only after whitening occurs. The cavities result from a translocation of moisture within narrow regions of the frozen crumb, but just how the new interfaces form is less certain. In most respects the phenomenon is analogous to freezer burn in other commodities, even though a loss of total moisture is not incurred by the loaf of bread. Bleaching or whitening of *surfaces* of frozen bakery products (freezer burn) does occur occasionally, but usually this is due to faulty or broken packages that permit surface dehydration. Internal whitening similar to the ring formation in bread has been observed by the present authors in frozen baking-powder biscuits and in an infrequent sample of frozen cake.

Consumers would almost certainly discriminate against bread with well-developed white rings. The abnormal appearance would also be accompanied by undesirable increases in crumb firmness, because conditions forming white rings lead to undue loss of softness (10). The ring defect is easily avoided in commercial practice by adherence to storage temperatures of 0°F. (−18°C.) or below, unless bread is to be stored for periods shorter than 2 weeks or longer than 3 to 4 months. The rings thus are a useful index of improper storage. Wide clear zones outside the rings indicate that bread was appreciably stale before freezing. Wide white rings indicate a large loss of moisture from a loaf during storage because of inadequate packaging.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Miss Kathryn M. Eremia of this laboratory for production of the laboratory bread used in these experiments. The cooperation of the Fairfax Bread Company, San Francisco, California, by providing the freshly baked commercial bread used in these studies, is also much appreciated.

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GRAIN STORAGE STUDIES

XXVI. Fungus Invasion and Deterioration of Wheats Stored at Low Temperatures and Moisture Contents of 15 to 18 Per Cent[1]

GEORGE C. PAPAVIZAS AND CLYDE M. CHRISTENSEN[2]

ABSTRACT

Samples of white wheat, not inoculated with fungi and inoculated separately with different species of fungi known to cause deterioration of stored grains, were stored at low temperatures and moisture contents of 15–18% and evaluated periodically for viability, brown germs, and percentage of seeds invaded by fungi. Inoculated and stored for 12 months at a moisture content of 15.0–15.5% and temperature of 5° and 10°C., the wheat did not decrease in viability and the germs remained light-colored. When stored at a moisture content of 16.0–16.5%, the wheat was extensively invaded by fungi at both 5° and 10°C., viability of the seed decreased, and the germs of many seeds became brown. Stored at a moisture content of 16–18% for 19 months, the seeds at −5°C. were not invaded by fungi, and remained sound and of high viability; at 5° and 10°C. many seeds were invaded by fungi, and the germs died and developed the brown color typical of sick wheat. Noninoculated controls which remained free of storage fungi remained sound and of high viability. The evidence suggests that wheat with a moisture content up to 16% may be stored without obvious deterioration for a year at a temperature of 10°C. or below, and wheat with a moisture content up to 18% may be stored safely for as long as 19 months at a temperature of −5°C.

Invasion of the germs of stored wheat by fungi is known to be a common cause of germ-damaged or "sick" wheat (3, 4, 5, 6).There is, however, little information available on the effect of low temperatures on such invasion and associated deterioration of the seed, although in the northern United States and in Canada much wheat is stored for several months to a year or more at temperatures below 20°C. It seemed worth while, therefore, to store samples of wheat at somewhat higher moisture contents and lower temperatures than those normally encountered in commercial storage, and to evaluate deterioration in seed quality under these conditions.

Materials and Methods

Wheat. A commercial sample of white wheat was used that had been grown in the low-rainfall region of eastern Oregon. The seeds were almost free of storage fungi, and had a viability of 98–99%.

Preparation of Samples. Quantities of wheat sufficient for each test

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were cleaned and were immersed and shaken in a 1% solution of sodium hypochlorite for 1 minute to eliminate most of such storage fungi as were present in the form of spores on the outside of the seed or as mycelium within the pericarps. The seed was then washed briefly in running tapwater, put in sterile paper bags, and dried to approximately 12% moisture content in a forced-draft oven at 32°C. This light treatment with sodium hypochlorite solution has no detectable effect on subsequent invasion by storage fungi (7). As indicated below, this treatment did not eliminate *Aspergillus glaucus* from all of the seeds.

The dried seed was placed in sterile bottles, sterile water was added to the controls, and sterile water plus spores of a single fungus was added to the inoculated ones, to achieve the desired moisture content. The bottles containing the seeds were kept for 5 days in a refrigerator at 5°C. and shaken occasionally, to permit even distribution of water. For each replicate, 50 g. of seed were placed in a sterile 1-qt. Mason jar equipped with rubber ring and screw cap. Those at a moisture content of 15.0–15.5% were kept for 8 days at room temperature, during which time it was assumed that some of the spores inoculated onto the seed would have germinated. In all other tests, the jars containing the seeds were placed immediately in incubators at the desired temperatures. Thermographs were placed in the incubators at various times during the storage period, and the maximum temperature variation recorded was ±2°C.

Fungi Used for Inoculum. Four members of the *Aspergillus glaucus* group—*A. amstelodami, A. repens, A. restrictus,* and *A. ruber*—as well as *A. candidus, A. flavus,* and *A. ochraceus* were used. These comprise the major storage fungi that have been isolated from numerous parcels of wheat that had deteriorated in commercial storage (3). Each fungus was inoculated alone; no combinations of different species were used.

Moisture contents were determined by the two-stage, air-oven method specified in *Cereal Laboratory Methods* (1) and are given on a wet-weight basis. Moisture contents were determined after the samples had been in the refrigerator for 5 days, and again at the end of the tests.

Viability of the seeds was measured by incubating 100–400 seeds of each sample on moist paper at room temperature for 4–6 days.

Percentage of Seeds with Dark Brown Germs. Pericarps were removed from the germs of 100 seeds, and the germs examined under a stereoscopic microscope with 10X magnification. Only those with the dark brown or black germs typical of sick wheat were rated brown.

Percentage of Seeds Internally Invaded by Storage Fungi. One hundred seeds were shaken in 1% sodium hypochlorite solution for 1 minute, rinsed in three changes of sterile water, placed on malt-salt agar in Petri dishes, incubated for 7–12 days at room temperature, then examined with a stereoscopic microscope and the fungi identified.

Results

Storage for One Year at 15.0–15.5% Moisture Content, Not Inoculated and Inoculated with Members of the Aspergillus glaucus *Group.* Viability of the seeds did not decrease, no germs became brown, and, with one exception, the seeds were not invaded by fungi (Table I). The single exception was the lot inoculated with *A. amstelodami*

TABLE I

FUNGUS INVASION, SEED VIABILITY, AND DEVELOPMENT OF BROWN GERMS OF WHITE WHEAT, NOT INOCULATED AND INOCULATED WITH MEMBERS OF THE ASPERGILLUS GLAUCUS GROUP, KEPT FOR 8 DAYS AT 25° ± 2°C. AND STORED FOR 12 MONTHS AT 15.0–15.5% MOISTURE CONTENT AND AT 5° AND 10°C.[a]

INOCULUM	FINAL MOISTURE CONTENT (ORIGINAL, 15.5%)	SEED VIABILITY	BROWN GERMS	FUNGI INVADING SEEDS[b]			
				A. amstelodami	*A. repens*	*A. ruber*	*A. restrictus*
	%	%	%	%	%	%	%
			Stored at 5°C.				
A. amstelodami	15.3	99	0	0	0	0	0
A. repens	15.3	95	0	0	0	0	0
A. ruber	15.1	98	0	0	0	0	0
A. restrictus	15.2	99	0	0	0	0	0
Not inoculated	15.4	100	0	0	0	0	0
			Stored at 10°C.				
A amstelodami	14.9	96	0	84	0	0	0
A. repens	15.1	99	0	0	0	0	0
A. ruber	15.2	98	0	0	0	0	0
A. restrictus	15.0	99	0	0	0	0	0
Not inoculated	15.1	99	0	0	0	0	0

[a] Each figure is an average of four replicates.
[b] As determined by culturing 100 surface-disinfected seeds on malt-salt agar.

and kept at 10°C.; 84% of these seeds yielded *A. amstelodami* at the end of the test. Presumably this invasion occurred during the 12-month storage period, since if any appreciable number of seeds had been invaded during the 8 days of incubation at room temperature before the seeds were placed at the lower temperature, those stored at 5°C. would have been similarly invaded. *A. amstelodami* will invade wheat seed stored at moisture contents of 13.0–13.6% at 25°C. (7), but further evidence is necessary to establish the minimum moisture content at which this fungus invades seed stored at 10°C. or below.

Storage for One Year at 16.0–16.5% Moisture Content, Not In-

oculated and Inoculated with Members of the Aspergillus glaucus *Group.* At 5°C. viability of the noninoculated controls remained above 90%, and none of the germs became brown. Viability of the inoculated seed ranged from 52 to 65%, and 21–41% of the germs were brown. At 10°C., viability of the inoculated seeds ranged from 12 to 27%, and 28–58% of the germs were brown. The noninoculated controls did not remain free of fungi; 18% became invaded by *A. amstelodami* and *A. repens,* viability decreased to 78%, and 4% of the germs were brown (Table II). Evidently storage at 5°–10°C. at a moisture content of 16.0–16.5% for a year may result in appreciable deterioration.

TABLE II

FUNGUS INVASION, SEED VIABILITY, AND DEVELOPMENT OF BROWN GERMS OF WHITE WHEAT, NOT INOCULATED AND INOCULATED WITH MEMBERS OF THE ASPERGILLUS GLAUCUS GROUP, AND STORED FOR 12 MONTHS AT MOISTURE CONTENTS OF 16.0–16.5% AND AT 5° AND 10°C.[a]

INOCULUM	FINAL MOISTURE CONTENT (ORIGINAL, 16.5%)	SEED VIABILITY	BROWN GERMS	FUNGI INVADING SEEDS[b]			
				A. amstelodami	*A. repens*	*A. ruber*	*A. restrictus*
	%	%	%	%	%	%	%
			Stored at 5°C.				
A. amstelodami	16.2	53	22	97	0	0	0
A. repens	16.1	62	21	0	66	0	0
A. ruber	16.4	65	27	0	0	77	0
A. restrictus	16.0	52	41	62[c]	12[c]	0	5
Not inoculated	16.4	94	0	2[c]	0	0	0
			Stored at 10°C.				
A. amstelodami	16.2	27	58	94	0	0	0
A. repens	16.0	23	47	0	89	0	0
A. ruber	16.0	18	28	0	0	95	0
A. restrictus	16.2	12	57	90[c]	0	0	15
Not inoculated	16.3	78	4	12[c]	6[c]	0	0

[a] Each figure in the table is an average of four replicates.
[b] As determined by culturing 100 surface-disinfected seeds on malt-salt agar.
[c] Invasion apparently resulting from inoculum originally present in the seed and not eliminated by the treatment with sodium hypochlorite solution at the beginning of the test.

That the fungi actually grew during storage at 5°C. is shown by the fact that sporophores of *A. glaucus* had developed on the germ ends of many of the inoculated seeds, as shown in Fig. 1.

Storage for One Year at 17% Moisture Content, Not Inoculated and Inoculated with Aspergillus candidus, A. flavus, *and* A. ochraceus. These fungi, prevalent in many samples of commercially stored wheat in which sick wheat has developed (3), require a higher moisture content for growth than do members of the *A. glaucus* group (2, 4, 5). At 5°C., viability decreased and brown germs developed only in the lot inoculated with *A. flavus* (Table III). The other lots remained sound and of high viability. At 10°C., viability of the inoculated lots

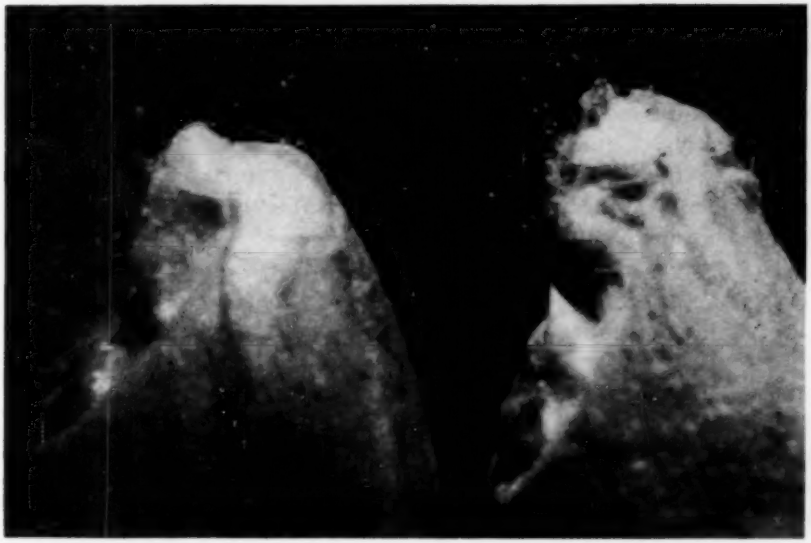

Fig. 1. Sporophores of *Aspergillus glaucus* growing from germ-end of wheat seed stored for 9 months at 16% moisture and 5°C.

ranged from 27 to 75%, and 2–25% of the seeds had brown germs. Viability of the controls remained high (84%) and no brown germs were found, although *A. glaucus* was recovered from 12% of the control seeds.

Most of the inoculated samples became infected with *A. glaucus*

TABLE III

FUNGUS INVASION, SEED VIABILITY, AND DEVELOPMENT OF BROWN GERMS OF WHITE WHEAT, NOT INOCULATED AND INOCULATED WITH ASPERGILLUS CANDIDUS, A. FLAVUS, AND A. OCHRACEUS AND STORED FOR 12 MONTHS AT 5° AND 10°C.[a]

INOCULUM	MOISTURE CONTENT		SEED VIABILITY	BROWN GERMS	FUNGI INVADING SEEDS[b]			
	Original	Final			*A. candidus*	*A. flavus*	*A. ochraceus*	*A. glaucus*[c]
	%	%	%	%	%	%	%	%
	Stored at 5°C.							
A. candidus	16.5	16.3	94	0	26	0	0	18
A. flavus	17.2	16.9	78	7	0	93	0	10
A. ochraceus	16.9	16.8	96	0	0	0	79	0
Not inoculated	17.0	16.9	95	0	0	0	0	2
	Stored at 10°C.							
A. candidus	16.5	16.4	49	25	33	0	0	70
A. flavus	17.2	17.0	27	23	0	87	0	18
A. ochraceous	16.9	16.8	75	5	0	3	89	17
Not inoculated	17.0	16.9	84	0	0	2	0	12

[a] Each figure in the table is an average of four replicates.
[b] As determined by culturing 100 surface-disinfected seeds on malt-salt agar.
[c] Invasion apparently resulting from inoculum originally present in the seed and not eliminated by the treatment with sodium hypochlorite solution at the beginning of the test.

during the storage period; therefore it is not known whether the reduction in viability and increase in brown germs were caused by *A. candidus, A. flavus,* and *A. ochraceus* acting alone, or whether damage to the seed was a product of combinations of *A. glaucus* with these species of fungi. In any case, the data again show that seed originally lightly invaded or contaminated by common storage fungi may undergo appreciable deterioration during storage for 1 year at a temperature of 10°C.

Storage for Nineteen Months at Moisture Contents of 16.6 and 18.2% and at −5°, 5°, and 10°C., Not Inoculated and Inoculated with Aspergillus candidus.

At −5°C., viability of the seed remained high in both the noninoculated and inoculated samples, and no brown germs developed, although a few of the inoculated seeds yielded *A. candidus.* At 5° and 10°C. the seeds originally at 16.6% moisture content decreased to a moisture content of 15–16%. Viability did not decrease and no brown germs developed. Viability decreased drastically, however, in the seed stored at 18.2% moisture content, and from 34 to 63% of the seeds developed brown germs. Both the noninoculated seeds and those inoculated with *A. candidus* and stored at 18.2% mois-

TABLE IV

FUNGUS INVASION, SEED VIABILITY, AND DEVELOPMENT OF BROWN GERMS OF WHITE WHEAT, NOT INOCULATED AND INOCULATED WITH ASPERGILLUS CANDIDUS, AND STORED FOR 19 MONTHS AT −5°, 5°, AND 10°C.[a]

TREATMENT	MOISTURE CONTENT		SEED VIABILITY	BROWN GERMS	FUNGI INVADING SEEDS[b]	
	Original	Final			*A. candidus*	*A. glaucus*[c]
	%	%	%	%	%	%
	Stored at −5°C.					
Inoculated	16.6	16.2	97	0	5	0
Not inoculated	16.6	16.1	98	0	0	0
Inoculated	18.2	18.1	97	0	10	0
Not inoculated	18.2	18.0	99	0	0	0
	Stored at 5°C.					
Inoculated	16.6	14.9	95	1	29	0
Not inoculated	16.6	15.5	98	0	0	1
Inoculated	18.2	18.0	39	34	0	99
Not inoculated	18.2	18.2	21	39	0	100
	Stored at 10°C.					
Inoculated	16.6	16.0	98	0	30	0
Not inoculated	16.6	15.6	98	0	0	0
Inoculated	18.2	18.1	24	56	0	100
Not inoculated	18.2	18.0	0	63	0	100

[a] Each figure in the table is an average of four replicates.
[b] As determined by culturing 100 surface-disinfected seeds on malt-salt agar.
[c] Invasion apparently resulting from inoculum originally present in the seed and not eliminated by the treatment with sodium hypochlorite solution at the beginning of the test.

ture content and 5° and 10°C. yielded *A. glaucus* from 99–100% of the seeds. Presumably this invasion resulted from inoculum originally present in the seeds and not eliminated by the treatment with sodium hypochlorite solution at the beginning of the test. It seems very probable that even where *A. candidus* was inoculated onto the seeds, the major cause of reduction in viability and development of brown germs during storage was *A. glaucus* (Table IV).

Discussion

The results suggest that wheat with a moisture content of 15.0–15.5%, even if moderately invaded by storage fungi, will remain sound and high in viability for as long as a year when stored at 5°–10°C. At moisture contents of 16–17%, little damage was caused by any of the fungi during storage for a year at 5°C., but considerable damage resulted at 10°C., and at 18% moisture content damage was severe in the seed stored at both 5° and 10°C. for 19 months. At moisture contents of 16–18%, members of the *A. glaucus* group became established in the seed, whether the seeds were or were not inoculated with this or other species of storage fungi. This invasion, apparently from inoculum naturally present on even the select seed in these tests, resulted in drastic decreases in viability and development of brown germs.

It has been shown that stored wheat may be invaded by various fungi at moisture content below those considered safe for long-time storage (7). The present tests indicate that the moisture content which permits damaging invasion of seed by storage fungi is a function of both time and temperature, and that as the temperature is decreased the moisture content may be increased without danger of deterioration. Wheat containing 15.0–15.5% moisture apparently can be stored at 5°C. for a year without damage, and wheat containing 16–18% moisture apparently can be stored at −5°C. for as long as 19 months without invasion by storage fungi, decrease in viability, or development of brown germs.

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PHYSICAL PROPERTIES OF NOODLES[1]

TORU SHIMIZU, HIDEAKI FUKAWA, AND AKIYOSHI ICHIBA[2]

ABSTRACT

Typical noodles from Japanese domestic wheat flour exhibited instantaneous elasticity of the order of 10^5 dynes/cm^2, a viscous flow of about 10^8 poises, and a retarded elasticity under constant load. The elastic modulus decreased with increase in temperature, which indicates that both entropy and internal energy changes occurred when noodles were deformed.

The elastic modulus, breaking energy, and stress relaxation increased with increases in crude protein content and boiling time. The gluten network plays an important role in a gelatinized and denatured dough.

Noodles are one of the important staple foods of Japan. Essentially, they are boiled dough strings made from soft wheat and salted water. The physical properties of wheat flour doughs and the development of apparatus for measuring them have been reviewed by Bailey (2) and more recently by Greup and Hintzer (4). Cunningham and Hlynka (3), Hlynka (5), and Hlynka and Anderson (6) have carried out comprehensive studies of wheat flour doughs under constant deformation and also of the structural relaxation of dough. There is little information, however, on the physical properties of dough that has been gelatinized and denatured, as is the case with noodles. The purpose of the present studies has been to investigate the rheology of these sys-

[1] Manuscript received March 25, 1957.
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tems using flours of different type and protein content which were boiled for various lengths of time.

Materials and Methods

A U. S. Western white wheat and a Japanese wheat were milled to 60% extraction on a Buhler experimental mill. The respective flours contained 7.1 and 9.2% protein, and 0.42 and 0.43% ash, on a 14% moisture basis.

In preliminary studies, noodles made from Japanese wheat flour were used. For additional studies of the relationship between the physical properties of noodles and flour protein content, two series of "flours" of various protein content were prepared. Gluten was separated from the starch of the Western white and Japanese flours by washing with tapwater. The gluten was then freeze-dried and crushed to pass through a 70-mesh sieve. The starch was recovered and dried *in vacuo* at about 40°C. By adding starch or gluten to the original flours, a series of seven samples of Western white flour were prepared with protein contents of 5.7, 6.4, 7.1, 7.8, 8.3, 8.8, and 9.3%. Similarly, seven samples of Japanese flour were prepared with protein contents of 7.1, 7.8, 8.3, 8.8, 9.3, 10.0, and 10.7%.

Noodles were prepared by mixing flour with 35.0% water containing 2.0% salt in a mechanical mixer for 5 minutes, sheeting 10 times,

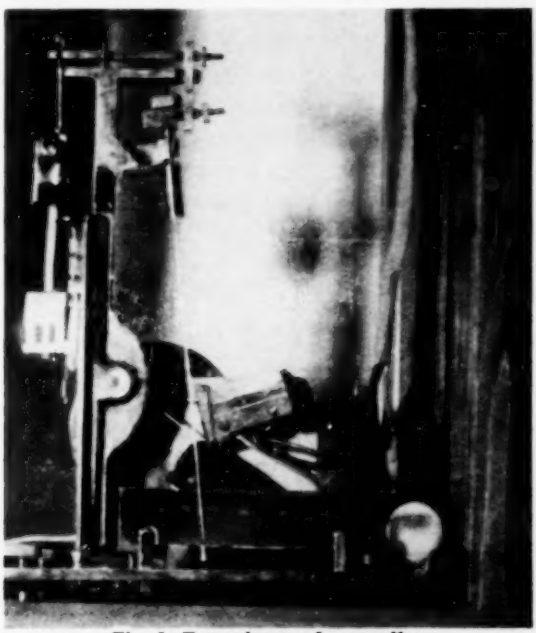

Fig. 1. Extensimeter for noodles.

and finally cutting the dough sheets into strips approximately 0.20 cm. by 0.25 cm. in cross-section. The noodles were then boiled and the physical properties of boiled noodles were measured 1.5 hours after boiling. At the same time, the rectangular cross-sectional area was calculated from measurements of each side, made by means of a microscope.

The load-extension relationship, elastic modulus, energy to the breaking point, and stress relaxation were measured using the instru-

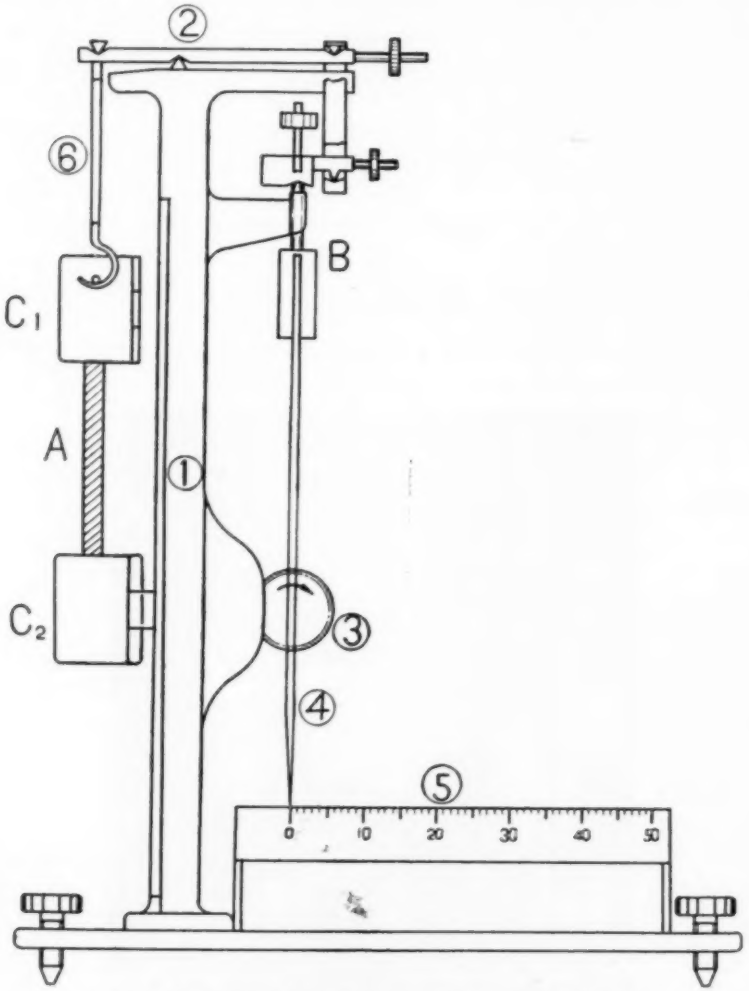

Fig. 2A. Schematic diagram of the apparatus. 1, stand; 2, beam; 3, drive gear; 4, compass needle; 5, scale; 6, hook; B, pendulum; C_1, C_2, chuck.

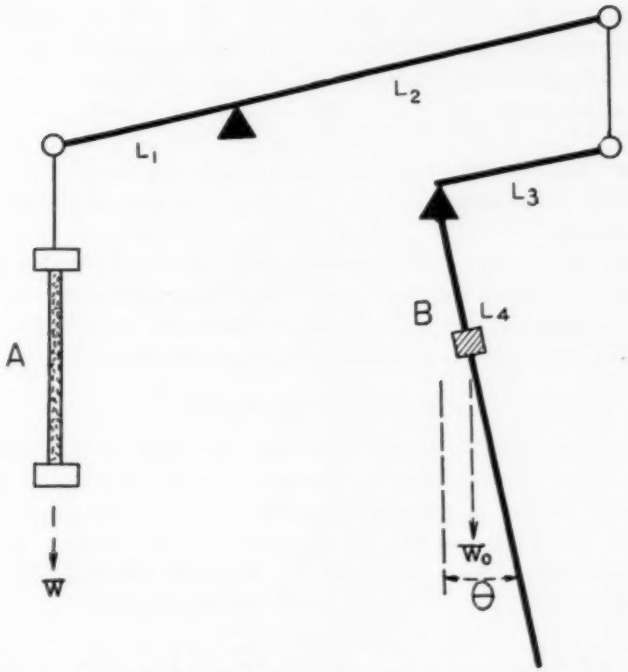

Fig. 2B. Mechanical relationships. The stress W on sample A is indicated through a lever system by deflection of the pendulum.

ment shown in Fig. 1. A schematic diagram of the instrument, which may be regarded as a type of beam balance, is shown in Figs. 2A and 2B. The left-hand side of the balance is attached to the test piece A, which is held between two chucks C_1, C_2. The stress is applied to the sample by load W. The right-hand side of the balance is linked to a pivoted pendulum B with the arms L_3 and L_4 forming a rigid right angle. A force applied at A is thus translated to a deflection of the pendulum from its horizontal position by angle Θ. When the left- and right-hand sides are in balance, then

$$L_1 W_1 = L_2 W_0 \frac{(L_4 \sin \Theta)}{(L_3 \cos \Theta)}$$

The term in parentheses is the contribution of the horizontal components of the pendulum regarded as a lever system. This relation can be rearranged to give

$$W = W_0 \frac{L_2 L_4}{L_1 L_3} \tan \Theta$$

Thus W is directly proportional to tan Θ and hence also to the scale reading, within limits.

The test piece of noodle is 5 cm. long. The scale in the instrument is 2 mm/g and the maximum load 50 g. The error caused by the movement of the pendulum for different load is:

Load, g.	10	20	30	40	50
Error, cm.	0.25	0.50	0.73	0.94	1.15

For the experiment of constant rate strain the lower chuck C_2 was driven by a synchronized motor (1 r.p.m.). The rate of elongation was 2×10^{-2}cm/sec.

In the case of noodle the relaxable stress was not so great (see Fig. 7). In these experiments the motor was stopped at a constant stress of 20% and the change of the load of the elapsed time was observed. The stress relaxation data were obtained approximately.

Results and Discussion

Behavior under Constant Load. For the study of the behavior of noodles under constant load, a four-parameter mechanical model as described by Alfrey (1, pp. 104–105) was selected to represent the rheological behavior of noodles. Figure 3 shows the essential features of this model: instantaneous elasticity G_1, retarded elasticity G_2, coupled with a damping viscosity η_2 and an actual flow viscosity η_3.

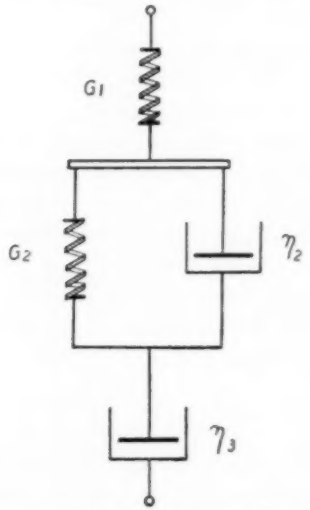

Fig. 3. Four-parameter model for substances exhibiting elasticity, retarded elasticity, and flow.

In practice, typical noodles from Japanese wheat flour (crude protein 9.3%, boiling time 12 minutes) were used in this experiment. The length of the sample noodle was 20 cm. and the cross-sectional area

was 0.13 cm². A load of 20 g. was applied at time 0 and removed after 360 seconds. Instantaneous elasticity, creep, and retarded elasticity were observed. Figure 4 summarizes the data in a strain-time diagram corresponding to the four-parameter model. The individual parameters were then evaluated from these data on the basis of the relationships described by Alfrey.

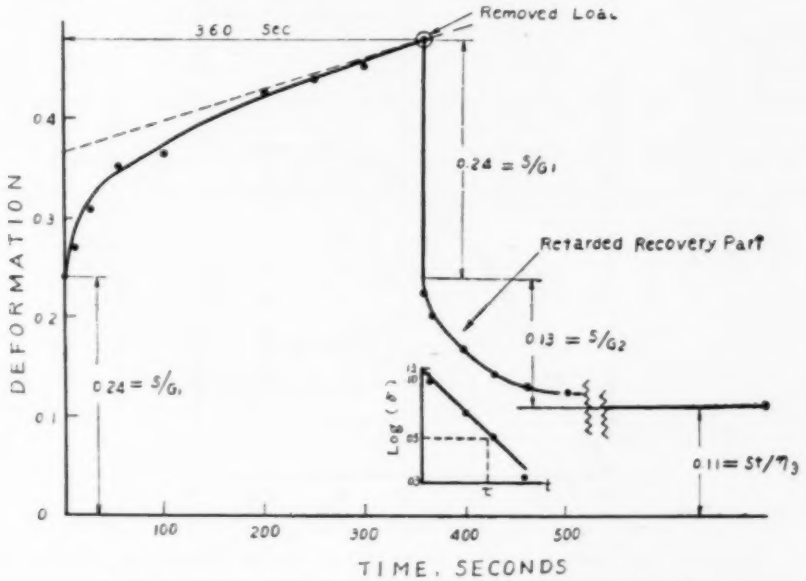

Fig. 4. Strain-time diagram of noodles under constant load.

Instantaneous elasticity G_1 was evaluated from the experimental value of $S/G_1 = 0.24$, and the value of stress S obtained from the applied load of 20 g. and the cross-sectional area of 0.13 cm.² of the test piece:

$$G_1 = \frac{20 \times 980}{0.13 \times 0.24} = 1.51 \times 10^5 \text{ dynes/cm}^2$$

The viscosity η_3 was calculated from the experimental value $(S/\eta_3)t = 0.11$, using the value of stress already given, and time of 360 seconds:

$$\eta_3 = \frac{20 \times 980 \times 360}{0.13 \times 0.11} = 4.9 \text{ poises}$$

The retarded elasticity was evaluated from the experimental value $S/G_2 = 0.13$ and the known value of stress to give

$$G_2 = 1.2 \times 10^6 \text{ dynes/cm}^2$$

The retardation time τ which is the ratio G_2/η_2 was evaluated

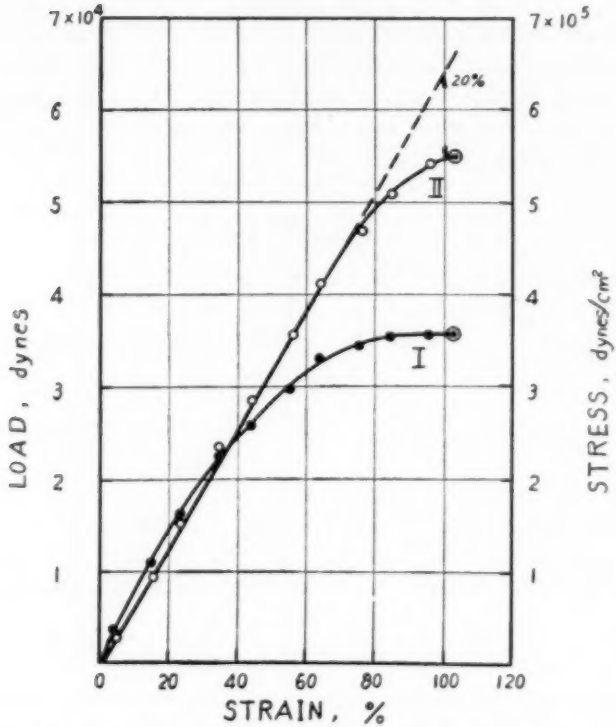

Fig. 5. I, load elongation curve of typical sample of noodles. II, stress-strain relationship calculated from curve I.

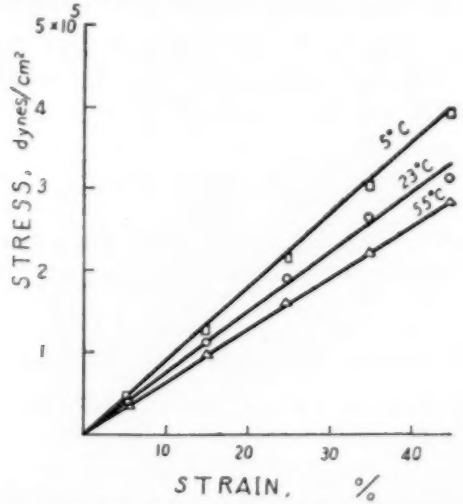

Fig. 6. Stress-strain relationship for noodles at 5°, 23°, and 55°C.

graphically in the manner described by Alfrey (1, pp. 58, 178) from the creep recovery part of the curve.

Namely, when the logarithm of the retarded recovery part ($\gamma - S/G_1 - St/\eta_3$) was plotted to the time, the value τ obtained was:

$$\tau \simeq 1 \text{ minute}$$

and

$$\eta_2 \simeq 7 \times 10^7 \text{ poises}$$

Thus, it has been shown that the noodle exhibited an instantaneous elasticity of the order of 10^5 dynes/cm^2, a viscous flow of the order of 10^8 poises, and a retarded elasticity which has a retardation time of about 1 minute.

Behavior under Constant Rate of Elongation and the Change in Elastic Modulus Temperature. The load-elongation curve of typical

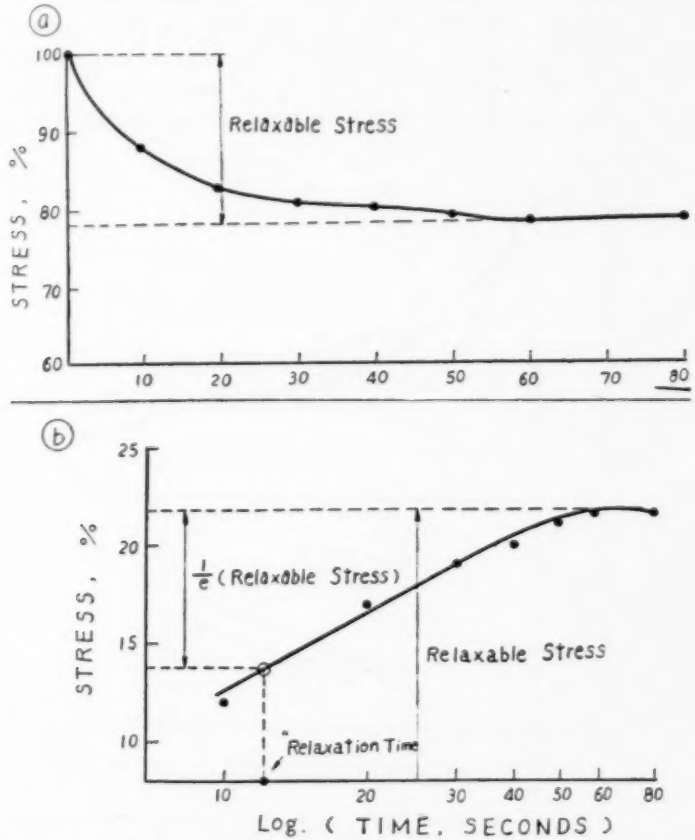

Fig. 7. a, Actual relaxation curve; b, linear transformation of relaxation showing evaluation of relaxation time.

noodles and the stress-strain relationship, which was calculated from it by correcting the load to a unit cross-sectional area, assuming an incompressible body, are shown in Fig. 5. The data show that with this instrument the stress-strain relationship of noodles was linear until the elongation of the noodles exceeded 60 to 70%. The elastic modulus was calculated from the slope of the linear part of the stress-strain curve. The energy up to the breaking point could be calculated from the area under the load-extension curve and the volume of the test piece.

Stress-strain relationships of the sample at different temperatures are shown in Fig. 6. The test piece, previously cooled by ice, was held to the chucks in a closed cabinet cooled by ice. After 10 minutes the elongation data were obtained. In this case the experimental temperature was about 5°C. Similarly, the data for about 55°C. were obtained by heating with infrared rays. The data for 23°C. (room temperature) were used as the control for this experiment. The elastic modulus decreased slightly with increase in temperature. Thus, it is suggested that a change of entropy and of internal energy both occur

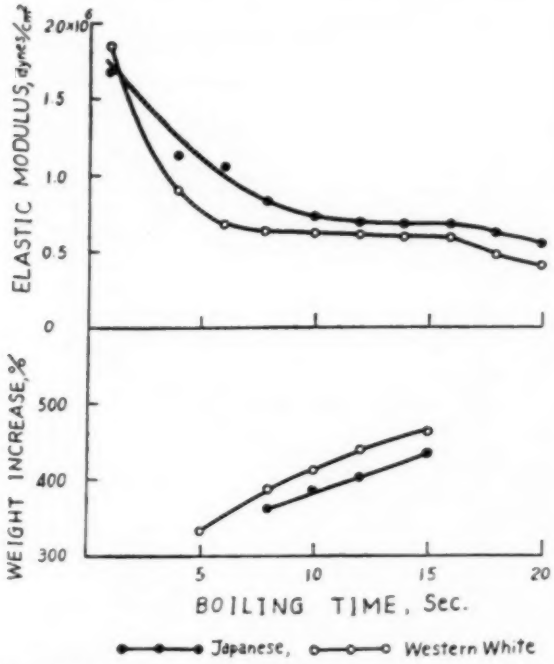

Fig. 8. Relationship between boiling time, weight increase, and elastic modulus at constant crude protein content.

when noodles are deformed. Owing to the many polar groups in noodles, many kinds of bonds would be broken upon deformation.

Behavior under Constant Elongation. Relaxation data were obtained by plotting the relation of the stress to time at constant elongation (1.0 cm.). Typical data are shown in Fig. 7, a.

The internal stress of noodles decreased rapidly at first, then more slowly, and finally remained at a constant unrelaxable stress of 70–80% after 60 seconds. When the data were plotted as relaxable stress against logarithm of time, the decay of stress was linear (Fig. 7, b).

This is the same type as the stress relaxation of dough which has been reported by Hlynka and Anderson (6). It has been explained by the generalized Maxwell model consisting of an array of Maxwell units in parallel. This puts a limitation on the use of a single relaxation time, but for the convenience of summarizing the data and of comparing with simple Maxwellian relaxation, the time in which the relaxable stress decayed to 1/e was adopted and termed apparent relaxation time.

Relationship between Boiling Time, Weight Increase, and Elastic Modulus under Constant Crude Protein Contents. Figure 8 shows the

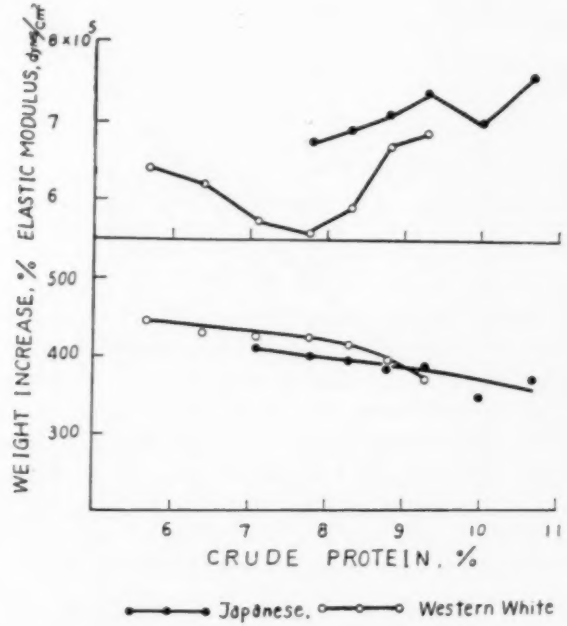

Fig. 9. Relationship between protein content, weight increase, and elastic modulus. (Boiling time, 12 minutes.)

relation between the boiling time of noodles, on the one hand, and the elastic modulus and percent swelling on the other. The swelling weight increased progressively with boiling time. In contrast, the elastic modulus remained constant over a broad range. It was thought that this range might be changed by protein content, but perhaps the formation of gel structure of noodles is completed in this range.

Relationships between Protein Content and Physical Properties. Relationships between protein content, weight increase, and elastic modulus at constant boiling time are shown in Fig. 9. Though the swelling weight decreased with increase in protein content, the elastic modulus increased.

The relationship between protein content and energy up to the breaking point is shown in Fig. 10. It appears that the energy increases with the increase in protein content.

Relationship between protein content and stress relaxation is shown in Fig. 11. In this case also, "relaxation time" increased with

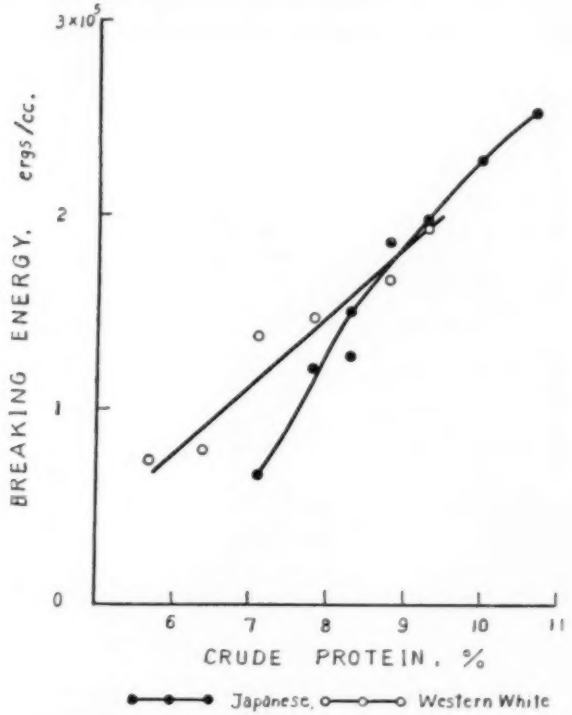

Fig. 10. Relationship between protein content and energy to the breaking point. (Boiling time, 12 minutes.)

increase in protein content. At the same protein level a larger relaxation time was exhibited by Western white wheat flour than by Japanese wheat flour.

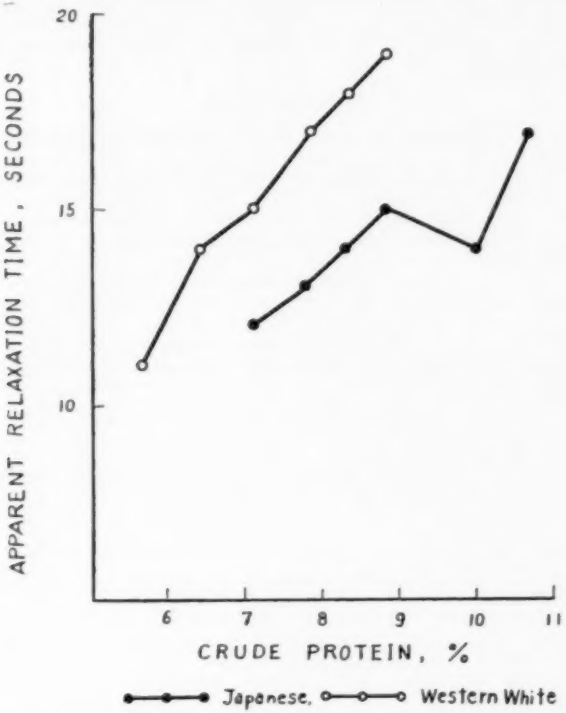

Fig. 11. Relationship between protein content and apparent relaxation time. (Boiling time, 12 minutes.)

General Discussion

Noodles consist of gelatinized starch and denatured protein. Although a four-parameter model adapted to approximate the physical behavior and properties of noodles under constant load gives a similar load-extension curve to the Brabender Extensogram® for dough, the elastic modulus and the viscosity of noodles are about 10 to 100 times greater than those of dough. The moisture content of noodles is 400–500% (dry basis) and is far higher than that of dough. It is supposed, therefore, that the magnitude of the physical properties of noodles is due to the gelatinized starch, although protein content has a great influence on the mechanical behavior of the system.

Studies are being undertaken of the manner in which the three-dimensional network of gluten changes in the boiling process.

Acknowledgment

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THE EFFECT OF MECHANICAL DAMAGE TO WHEAT GRAINS DURING SCOURING ON THEIR SUBSEQUENT ABSORPTION OF WATER DURING WASHING[1]

J. D. CAMPBELL[2]

ABSTRACT

Scouring of wheat causes damage to the pericarp, permitting localized entry of water immediately beneath it during immersion of the grain for 1 minute as in washing prior to milling. The locus is at the beard end of the dorsal surface of the grain; from this point the water may travel by capillarity along the back of the grain immediately beneath the pericarp for a distance depending on the extent of the damage. This distance, which may be measured by means of staining, is related to the weight of water picked up by the grain during the immersion. The additional water picked up as a result of the damage is held during a brief period mainly by the bran coats at the beard end, but redistribution between the germ and beard halves of the grain occurs rapidly. The rate of subsequent moisture penetration into the endosperm is increased, in the case of scoured grain, only in the peripheral dorsal region of the endosperm.

The pick-up of water during 1 minute's immersion varied from 3.0 to 4.9% of the initial weight of the grain in various samples each of English Atle, Canadian Marquis, and also imported Manitoba wheats. This wide range is due to variation in extent of pericarp damage and in initial moisture content of the samples.

It is a well-known but unexplained fact that different parcels of any given type of commercial wheat pick up and retain different proportions of water during the brief period of immersion involved in commercial wheat washing. The differences may be considerable, resulting in complication of control of the subsequent milling processes. Previous work (3, 4, 9) has shown how mechanical handling of wheat, and in particular scouring, damages the bran by splitting the pericarp, resulting in a greater pick-up of water during commercial wheat washing. This damage is particularly pronounced at the beard and germ ends of the grain, and along the dorsal surface. Jones and Baker (7) found that imported wheats, as a result of commercial handling, contained varying proportions of kernels with germs exposed due to partial removal of the bran. This exposure could also be produced by means of laboratory scouring treatment. Since germ comprises about 3% by weight of the grain, and is known (1) in the isolated condition to pick up 40% by weight of water in 1 hour (shorter periods of immersion have not been studied), it seemed conceivable that a difference equal to 3% of 40%, i.e. 1.2%, in pick-up by grain during immersion might occur as a result of different degrees of exposure of the germ.

[1] Manuscript received September 3, 1957.

[2] The Research Association of British Flour-Millers, Cereals Research Station, St. Albans, England.

These considerations prompted the present investigation of factors affecting the weight of water absorbed by wheat during washing. However, as will be shown, attention necessarily became focused on the beard end of the grain as the main site of the damage that affects water uptake during brief periods of immersion.

Materials and Methods

Wheat. The following wheat samples were available for examination: (1) three samples of English-grown combine-harvested Atle wheat; (2) six samples of Nos. 1 to 4 Manitoba Northern wheats taken from the holds of ships at various English ports; (3) samples of Manitoba wheat, Export Standards, sent direct to St. Albans from Canada, by courtesy of the Board of Grain Commissioners for Canada; and (4) samples of Marquis wheats, similarly sent in connection with the 1953 Collaborative Varietal Tests.

Scouring. The wheat samples were passed through a Wolf laboratory scourer (fitted with an indented metal casing).

Method of Determination of Water Pick-up by Grain. Water pick-up was determined by weighing 100 g. of grain into two muslin net bags. After immersion in water for 1 minute the bags were suspended from the center spindle of the laboratory centrifuge and centrifuged for 30 seconds. The suspension of the bags was so arranged that during the centrifugation they were flung against the walls of the wire basket, thus ensuring the maximum centrifugal effect. The grain was tipped out and weighed to determine the increase in weight due to water pick-up.

To measure the water pick-up in grains which had been individually partially sealed off from water entry, twenty grains were selected at random, weighed, and placed in two small wire-mesh baskets, ten grains in each. After immersion in water for 1 minute the baskets were suspended from the central spindle of the centrifuge by means of two wires of such length that the orbit followed by the grain was 13 in. in diameter, which provided a centrifugal force of 200 × gravity at the operating speed of 1030 r.p.m. The samples were centrifuged for 30 seconds in the normal way. The baskets were then quickly transferred to glass-stoppered cylinders to reduce evaporative losses, and weighed.

Sealing of Grains with Localized Waterproofing Treatment. To make a comparison between water uptake at the germ and beard ends of the grain during 1 minute's immersion, a waterproof coating was applied to each end of the grain in turn. For applying the localized waterproofing treatment, Durofix (a cellulose-amyl acetate cement) was found to be a suitable agent. Localized sealing of grain areas had pre-

viously been applied by Swanson and Pence (10) and by Ugrimoff (11), but only in the case of grains immersed for relatively long periods.

Moisture Determinations on Cut Grains by Means of Density Measurements. Individual grains, both of unscoured and scoured grains, were cut transversely into halves after different short periods of lying, following immersion and centrifuging. The total moisture content of each half of the grain was determined by applying a method previously developed (8) for estimating moisture contents of dissected endosperm particles by means of microdensity determinations. The present application depends upon the fact previously shown (8) that after a period of 72 hours at 20°C., uniform distribution of moisture throughout the grain has been attained; a microdetermination on a particle of endosperm dissected from, say, the central cheek may therefore be presumed to indicate the moisture content of the entire half-grain. The half-grain was accordingly stored for 72 hours at 20°C. before the dissection, loss of moisture being prevented by cutting and keeping the specimens under the water-saturated oil in which grains are normally immersed during dissection of endosperm particles.

Tracing the Extent of Water-Travel beneath the Pericarp. In scoured grains placed with their beard ends in contact with water, rapid movement of water in the pericarp layers along the back of the grain towards the germ end could be seen under low-power magnification. This movement was made more readily visible by suitable staining treatment. Of several stains tried, the most suitable was a 0.1% solution of methyl orange in methanol. A grain was stained by holding it, beard end downwards with the beard just in contact with the stain, for 1 minute. Any excess of stain adhering to the grain was wiped off with blotting paper.

For recording the extent of staining, the length of the grain from the beard end to the germ end was subdivided mentally into six regions. A grain was allotted a score of 1 if the stain had moved through the first zone (counting from the beard end), 2 if it had moved through the first and second zones, and so on. Estimations were made to the nearest half-zone (equivalent to about 0.5 mm.) on six grains selected at random from each sample.

Adjustment of Initial Moisture Content. In preparation for a study of water pick-up in relation to the initial moisture content of the grain, the moisture content of a sample of No. 2 Manitoba Northern wheat at 13.6% moisture (as received), was adjusted to levels in the range 14–23% and the wheat allowed to stand for 7 days, prior to determination of the weight of water picked up during immersion for 1 minute.

Results

Water Pick-up Following Scouring. The weight of water per 100 g. of English Atle and Canadian Marquis wheats following scouring and 1 minute's immersion in water was as follows:

Number of Scourings	*Percent of Initial Weight*	
	Atle	*Marquis*
None	1.88	3.42
One	3.09	4.50
Six	4.0	5.55

The initial moisture contents of these wheats were 13.0 and 13.2% respectively.

The data of Jones (6) would indicate that with centrifugal effects of the order (200) used in the present experiments, the pick-up by Manitoba wheat following 1 minute's immersion should be about 5.4% of the initial weight of grain in the absence of appreciable evaporative loss. The fact that the present values are lower than this by about 0.8% is doubtless due to the occurrence of considerable evaporation under the conditions of centrifuging obtaining in these smaller-scale experiments. Naturally this difference does not invalidate the application of the method in comparing various samples of grain, since the effects of variation in extent of evaporation and of variation in centrifugal effect may be regarded as supplementary. Thus from the data of Jones (6) the present results for unscoured Marquis are equivalent to those that would be obtained with a centrifugal effect of 350 in the absence of evaporative loss. Jones also showed (6) that under comparable conditions English wheats pick up considerably less moisture than Manitoba during immersions of short duration, but this statement applies essentially to large-grained English wheats, and the present figure for the unscoured Atle wheat is perhaps unexpectedly low. Nevertheless the striking feature is the extent to which, with both wheats, a single scouring treatment has increased the water uptake by about 1%. Additional scourings produce a further increase, but at a smaller rate. The trend, therefore, does not resemble that known (7) to be characteristic of the extent of germ exposure following successive scouring treatments.

Water Pick-up in Relation to Initial Moisture Content of the Grain. The relationship between water pick-up and initial moisture content of the grain is shown in Fig. 1, which emphasizes also that the over-all pick-up is less in English than in Manitoba wheats. Furthermore there is a gradual reduction in water pick-up as the initial moisture content rises, and conversely a gradual increase in water pick-up

on subsequent drying back of the damped grain. Again when a sample of wet English wheat was dried back from 23 to 13% moisture content,

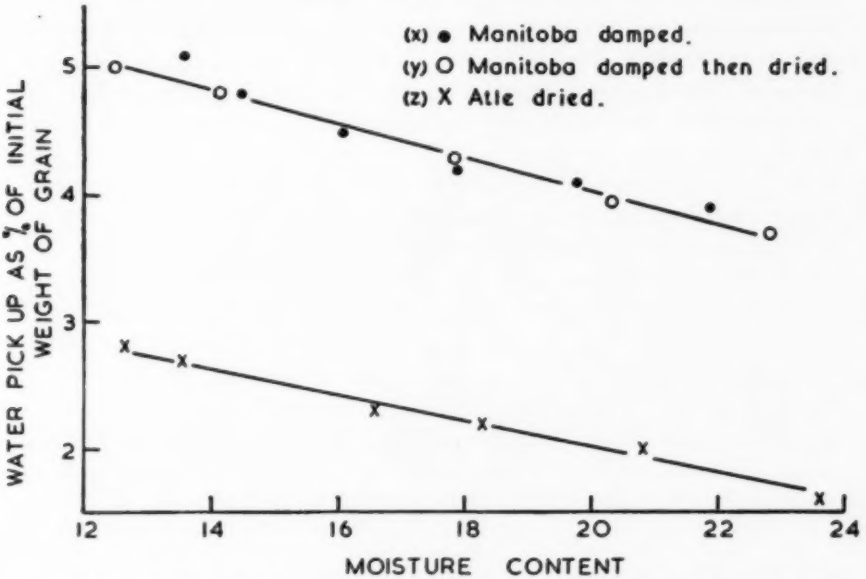

Fig. 1. The effect of variation in initial moisture content on pick-up of water during washing by: (x) Manitoba samples with initial moisture content increased to varying extents by preliminary damping; (y) Manitoba samples with initial moisture content decreased by drying after a preliminary heavy damping; (z) Atle samples with initial moisture content decreased by drying from a high "natural" level.

TABLE I

EFFECT OF SCOURING OF MARQUIS WHEAT ON THE UPTAKE OF WATER DURING 1 MINUTE'S IMMERSION OF GRAINS WITH LOCALIZED WATERPROOFING TREATMENT

TREATMENT	UPTAKE OF WATER BY GRAIN				
	Successive Scourings			Increase Due to One Scouring (B-A)	Increase Due to Six Scourings (C-A)
	None (A)	One (B)	Six (C)		
Nonwaterproofed	3.42	4.50	5.55	1.08	2.13
Germ-end-proofed	3.07	3.98	4.86	0.91	1.79
Beard-end-proofed	3.23	3.34	3.97	0.11	0.74
Reduction in uptake[a] due to waterproofing of					
Germ end	0.35	0.52	0.69	0.17	0.34
Beard end	0.19	1.16	1.58	0.97	1.39
Percentage reduction in uptake[b] due to waterproofing of					
Germ end				15.7	16.0
Beard end				89.8	65.2

[a] Expressed as a percentage of the initial weight of the grain.
[b] Expressed as a percentage of the increase in water uptake by the nonwaterproofed grains due to 1 and 6 scourings, respectively.

the weight of water pick-up, although appreciably less than in the Manitoba, was found to increase as the initial moisture content fell.

Water Pick-up by Grains with Localized Waterproofing Treatment. The effect of the application of localized waterproofing treatment is shown in Table I. The increased uptake due to scouring was largely prevented by waterproofing the beard ends, but was reduced by only 16% when the germ ends were waterproofed; thus the germ end of the grain, even when the germ is well exposed – as must have been the case after six scourings – contributes surprisingly little to the water uptake by the grain during immersion for 1 minute. This is in contrast to the behavior of wheat on immersion for long periods when (11) the entry through the bran near the germ is particularly heavy. Table I shows that the beard end is responsible for the greater part of the increase, due to scouring, in pick-up of water that occurs during washing.

Moisture Determination on Cut Grains. By way of controls in using the microdensity method to ascertain the moisture contents of respective half grains of variously treated specimens, dissections and density determinations were performed on cut grains, three determinations in each case being made. The results are recorded in Table II which

TABLE II

EFFECT OF SCOURING ON THE MOISTURE CONTENT OF CORRESPONDING GERM AND BEARD HALVES OF CANADIAN MARQUIS WHEAT (INITIALLY AT 13.2% MOISTURE CONTENT) FOLLOWING TRANSVERSE BISECTION AFTER DIFFERENT SHORT PERIODS FOLLOWING 1 MINUTE'S IMMERSION IN WATER

Time Interval between Centrifuging and Cutting	Moisture Content[a]				
	Uncut Grains	Half Grains			Difference between Beard and Germ Ends
		Beard Ends	Germ Ends	Average of Beard and Germ Ends	
minutes	%	%	%	%	%
Unscoured wheat					
4	16.0	15.9	15.9	15.9	nil
6	16.0	16.0	15.9	15.95	0.1
12	16.0	16.0	16.0	16.0	nil
Scoured wheat[b]					
4	16.8	17.4	15.9	16.65	1.5
6	16.7	17.2	16.1	16.65	1.1
12	16.9	17.0	16.7	16.85	0.3

[a] Estimated by means of microdensity determinations on particles of endosperm dissected from the central cheek after the grains or half grains had lain under oil for 72 hours at 20°C.
[b] Passed once through the laboratory scourer.

shows that the moisture content of the whole grain, after immersion and centrifuging, had increased by 2.8 and 3.6% respectively in the unscoured and scoured wheat samples. These increments expressed as percentages of the initial weight of the grain are 3.3 and 4.3% respectively and are thus comparable with the corresponding figures given in

the text which were obtained by a different method. Table II shows further that the additional water taken up by the scoured grain is entirely held in the beard end during the first 4 minutes after wetting, but it becomes distributed nearly equally between the two halves of the grain within 12 minutes.

Tracing the Extent of Water Travel beneath the Pericarp. Figure 2 shows the results of applying the staining method to wheat grains:

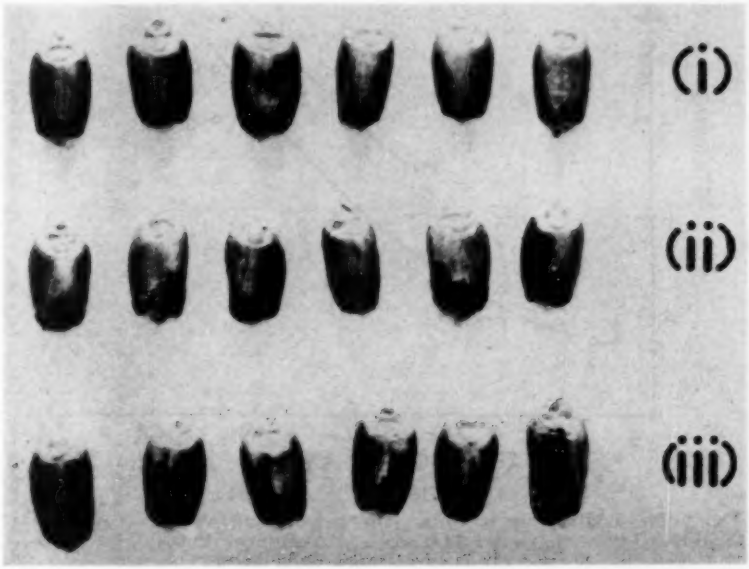

Fig. 2. The extent of stain penetration from the beard end of grains following: (i) no scouring; (ii) one scouring; (iii) six scourings.

unscoured (i), scoured once (ii), and scoured six times (iii). These variations in extent of staining were not shown when the germ ends of grains that had received different handling treatments were similarly dipped in the stain.

Numerous samples representing English Atle, Canadian Marquis, and imported Manitoba Northern wheats were examined by this method. The results indicated that the extent of staining as scored by the zoning system was related to the weight of water picked up following immersion during wheat washing (see Fig. 3). In all cases weight of water pick-up is based on an initial moisture content of 13%. The relationship between water pick-up and the initial moisture content of grain has been established experimentally in Fig. 1, and from this graph the water pick-up of a sample of wheat at any moisture content can be adjusted to a 13% basis. Figure 3 indicates that water pick-up

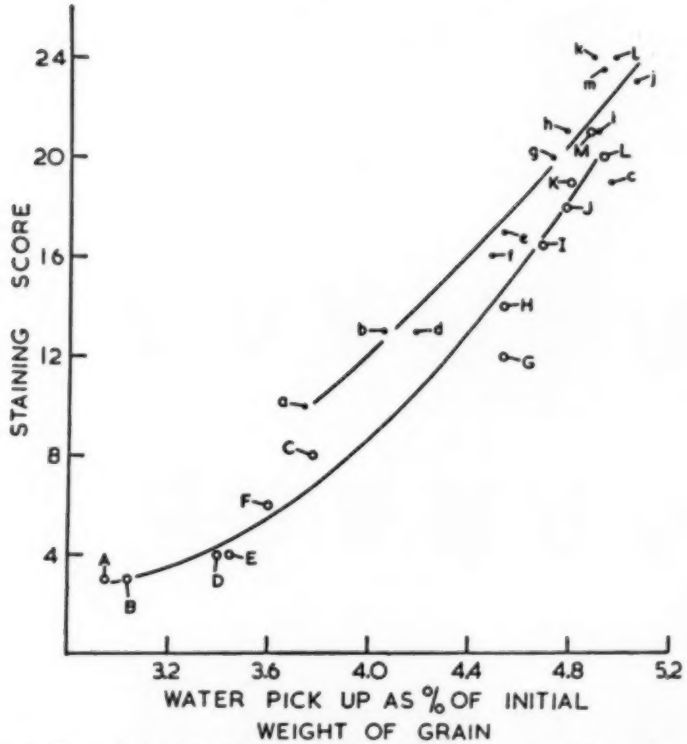

Fig. 3. The relationship between staining score and water pick-up in a series of English Atle and imported Marquis and Manitoba wheats. The lower curve (A–M) represents the water pick-up of unscoured wheats, and the upper curve (a–m) the water pick-up for the corresponding wheats after they were passed through the laboratory scourer.

increased with extent of damage to the pericarp caused by handling, and this increase was even more pronounced after the same samples had been passed through the laboratory scourer. English Atle and Export Standard Manitoba and Marquis wheats (A – F), which have been subjected to comparatively little commercial handling, show a much larger increase in water pick-up and staining score following scouring treatment, than do the commercial imported Manitoba wheats (G – M).

Rate of Moisture Penetration into the Endosperm of Scoured Grain. The rate of penetration of water during storage of the damped grain into different areas in the central cross-section of the endosperm of scoured Manitoba grain was measured by the method previously reported (2). These results are shown in Fig. 4 and can be compared with those (Fig. 2 of reference 2) for unscoured grains, as both wheats were damped to the same level of 17% moisture content (8). Any increase in

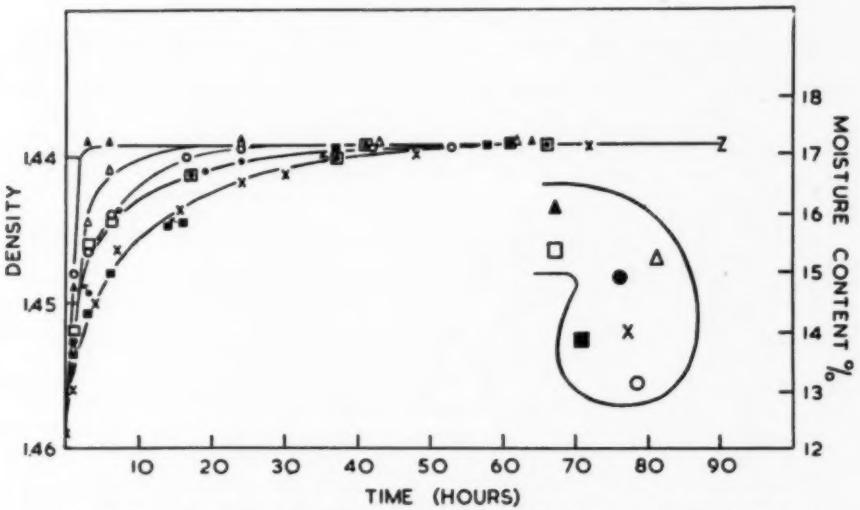

Fig. 4. Rate of penetration of moisture at 20°C. (68°F.) to various points in the central cross-section of damped, scoured Manitoba wheat as shown by the density of the endosperm at various times after damping. The experimentally determined density values have been adjusted for position by means of appropriate allowances as previously shown (reference 2, Fig. 2) before being plotted here. The point Z represents identical values obtained at 90 hours for endosperm from all parts of the grain.

rate of water penetration in the scoured wheat was confined to the endosperm near the bran along the back of the grain (Fig. 4). In this area it took 5 hours to reach moisture content equilibrium as against 15 hours in the unscoured wheat previously examined.

It is difficult to account for this increased rate of penetration. Hinton (5) has reported that the testa is the layer that restricts the rate of passage of water into the endosperm, but examination of scoured grains showed no apparent damage to the testa along the dorsal surface of the grain. At the same time it must be remembered that Hinton used a different technique: he brought liquid water into direct contact with the testa for periods of 30 minutes and upwards.

In the present work the time of immersion was only 1 minute. During this period liquid water would come into contact with the testa in the mechanically damaged grains only, and even then only in the dorsal area of the grain that absorbs stain most readily. Liquid water would apparently not come into contact at all with the testa of the unscoured, undamaged grain, in which contact with water presumably takes place only via the vapor phase. This distinction may offer some explanation of the different pattern of water movement in the dorsal and peripheral endosperm in scoured grains.

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EFFECTS OF STORAGE TEMPERATURE ON FIRMING OF CAKE CRUMB[1]

J. W. PENCE AND N. N. STANDRIDGE[2]

ABSTRACT

Firming of crumb of yellow layer, chocolate layer, angel food, and pound cakes, measured as force per fixed deformation on standard sections, became progressively more rapid at storage temperatures of 0°, 10°, 20°, 34°, and 70°F. (−18°, −12°, −7°, 1°, and 21°C.). The firming rates, in general, were rather small and seldom significantly different between adjacent temperatures because of both the slow rates of change in firmness and a relatively large variation in firmness of the starting material.

The firming rates of cakes were so small as to suggest that firmness changes might be of lesser concern in frozen or refrigerated storage of cakes than possible changes in flavor, moisture, or other aspects of texture. Freezing points of the cakes used fell between 3° and 8°F. (−16° and −13°C.). The retarding effect of lower temperatures on rate of firming of cake crumb explains in part their pronounced tolerance to variations in freezing and refrigeration conditions in commercial cake-freezing practice.

A well-known rule of thumb in the baking industry states that richer products freeze better than lean ones. Breads, for example, must be frozen and defrosted rapidly to avoid undue loss of their original crumb softness. Cakes, on the other hand, seem to tolerate a wide range of variation in freezing procedures without notable ill effects (3). The great differences in composition of these two types of products undoubtedly are responsible for their different behavior during freezing, but no suggestion as to possible resulting differences in firming mechanisms has so far been encountered in the literature.

Bread is known to become firm at increasingly greater rates as its temperature is reduced toward its freezing point (2, 6, 8 among others). Rapid freezing and defrosting thus lead to a minimum loss of softness in the original product. An increasing crystallinity of the gelatinized starch in bread crumb is most often associated with this crumb firming (1, 2, 9 among others). Partial inhibition of starch retrogradation, either by reduction of the starch content of breads or by use of ingredients that complex with starch molecules (1), decreases the rate of firming of bread crumb.

In foam cakes, such as angel food or chiffon, the actual starch contents are especially low so that a firming behavior different from that of bread can reasonably be expected. The same would be true

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for other cakes, though perhaps to a lesser degree. Cakes become firm with age at much slower rates than breads[3] (7), but none of the published articles, so far available to the authors, that discuss cake staling have described any effect of temperature on the rate at which cake crumb becomes firm. The present paper deals with a study of the influence of storage temperature on firming rates in cakes.

Materials and Methods

The cakes used were commercially produced and selected to represent the major basic types of cake normally encountered in retail operations. All were high-ratio or otherwise rich formulations that would be considered as high-quality representatives of their type.

The cakes were not iced and were obtained from a single retail bakery within 2 to 3 hours after baking. Individual 7-inch yellow and chocolate layers, whole pound cake loaves, and halves of 12-in. angel food cakes baked in tube pans were wrapped in MST 300 moisture-resistant cellophane immediately after cooling and placed in storage as soon as possible. Temperatures at the centers of the cakes became essentially equal to the storage temperatures within 3 to 6 hours after they were placed in the storage compartments. Temperatures of the storage rooms were maintained, automatically, well within a ±2°F. (1°C.) range throughout the storage periods.

Samples withdrawn from storage for determination of firmness were allowed to equilibrate at room temperature for 4 hours before measurements were made. Firmness readings were made with a Baker Compressimeter equipped with a pressure plate 1¼ in. (33 mm.) square on sections of crumb, cut to these dimensions and 1 in. in height by means of miter boxes. Readings were taken at 4 mm. of compression (unless otherwise noted) on four separate sections from each cake.

Results and Discussion

Comparison of Firmness Variability in Sheet Cakes and Circular Cakes. As layer cakes are often baked in sheet form, a preliminary investigation was made to determine whether large sheet cakes would be more satisfactory sample material than the smaller cakes 7 in. in diameter. Accordingly, six round layers and one-half of a sheet cake, originally measuring 16 by 24 in., from the same yellow layer cake batter were examined for uniformity of firmness. The sheet cake (16 by 12 in.) was cut into 20 equal-sized pieces. Then a sample 1¼ in. square and 1 in. in height was cut from the center of each piece. Four

[3] Kulp, K., Ponte, J. E., Jr., and Bechtel, W. G. Some factors that affect the staling of white and yellow layer cakes. Paper presented at 41st annual meeting, New York, 1956.

similar samples were cut from the center of each circular layer and an additional four samples were taken from the remaining peripheral portions of these layers. The firmness readings obtained from the small sample sections were then analyzed statistically.

The total variance of the values for the sheet cake used in this experiment was nearly 20 times as large as that for all the values from the six circular cakes. The average value for individual circular layers was significantly different ($P = 0.05$) when values either for the central samples or the peripheral samples were used as an estimate of error. The total variance was slightly larger for peripheral than central samples, but the difference between mean values was not significant. All subsequent determinations of firmness in layer cakes were therefore obtained from four samples cut from the center of small circular cakes.

Effects of Temperature on Firming in Different Kinds of Cakes. Storage temperatures used in these experiments were 70°, 34°, 20°, 10°, and 0°F. (21°, 1°, −7°, −12°, and −18°C.). Freezing points of the cakes used were all between 3° and 8°F. (−16° and −13°C.), so that only cakes stored at 0°F. (−18°C.) were actually frozen during storage. Firmness values were obtained on samples of each of the four kinds of cake immediately after receipt from the bakery, and the remainder were distributed into the several cold rooms in a prerandomized fashion. Samples were likewise withdrawn for firmness measurements according to a randomized schedule. At intervals of 2 to 3 days over a storage period of up to 11 days, firmness measurements were made on one of each kind of the cakes. The storage experiment itself was replicated three times in order to reduce the number of cakes to be handled at the start of each experiment and to distribute the effects of all sources of variability as widely as possible.

All cakes appeared to show a linear increase of firmness with time over the storage periods used, and the rates of increase in firmness of all but the pound cake were relatively small. Pound cakes were appreciably firmer than the other cakes at the start of the experiments and became firmer at greater rates. In fact, it was necessary to use a crumb compression of only 1 mm. for pound cakes in order to obtain readings for the duration of the storage experiments. A compression of 4 mm. was used for all the other cakes.

Results from the three storage experiments were treated separately, in that coefficients of regression for firmness on storage time were calculated for each type of cake at each temperature and for each replication of the experiment. All the regression coefficients for each type of cake in turn were then submitted to variance analysis. Mean

regression lines were used for graphical representation of the results in order to avoid confusion due to the large number of points that would have to be shown.

Figure 1 shows regression lines representing the average rates of increase in firmness of chocolate cakes at the various temperatures. Because of the rather large degree of variability in the sample material, only the lines for 20°, 10°, and 0°F. (−7°, −12°, and −18°C.) differ significantly in slope ($P = 0.05$) from that for 70°F. (21°C.), and

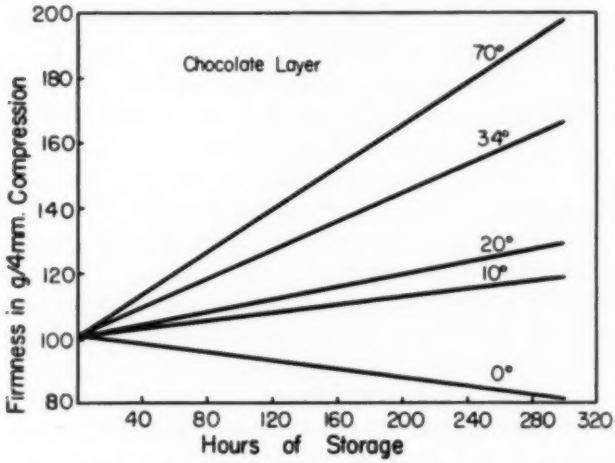

Fig. 1. Regression lines representing average rates of firming of chocolate cake layers at various temperatures (°F). Centigrade equivalents for the Fahrenheit temperatures shown are: 21°, 1°, −7°, −12°, and −18°C., respectively.

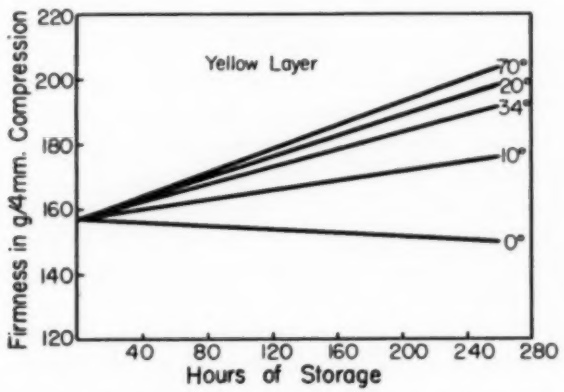

Fig. 2. Regression lines representing average rates of firming of yellow cake layers at various temperatures (°F.). Centigrade equivalents for the Fahrenheit temperatures shown are: 21°, −7°, 1°, −12°, and −18°C., respectively.

only the one for 0°F. (−18°C.) differs from the one at 34°F. (1°C.). All others do not differ significantly. The slope of the line at 0°F. (−18°C.) has a negative sign, but its value does not differ significantly from zero, so that the apparent softening of the cake during storage at this temperature is not statistically real.

Similar data for yellow cakes are shown in Fig. 2. In this series only the line for 0°F. (−18°C.) differs significantly from those at 70°, 34°, and 20°F. (21°, 1°, and −7°C.). All other differences are nonsignificant. The slope of the line for 0°F. (−18°C.) has a negative sign, but again it does not differ significantly from zero.

In Fig. 3, the lines for angel food cakes stored at 0° and 10°F. (−18° and −14°C.) differ significantly from the lines at the three higher temperatures, but not from each other. The three upper lines also do not differ significantly from each other.

For pound cakes, the line for 70°F. (21°C.) in Fig. 4 differs from the lines at 20°, 10°, and 0°F. (−7°, −14°, and −18°C.). The line for 34°F. (1°C.) differs from those at the two lower temperatures. No other significant differences are present.

Figure 5 is included to permit a ready comparison of firming rates at 70°F. (21°C.) for the two kinds of layer cake and the angel food cake. Pound cakes, of course, cannot be compared directly, because of the different scale used for their firmness evaluation, but their greater initial firmness and their more rapid rate of firming are indicated.

A major difficulty encountered during the course of the storage

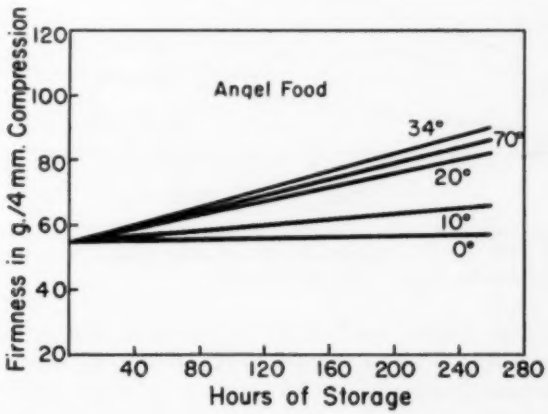

Fig. 3. Regression lines representing average rates of firming of angel food cakes at various temperatures (°F.). Centigrade equivalents for the Fahrenheit temperatures shown are: 1°, 21°, −7°, −12°, and −18°C., respectively.

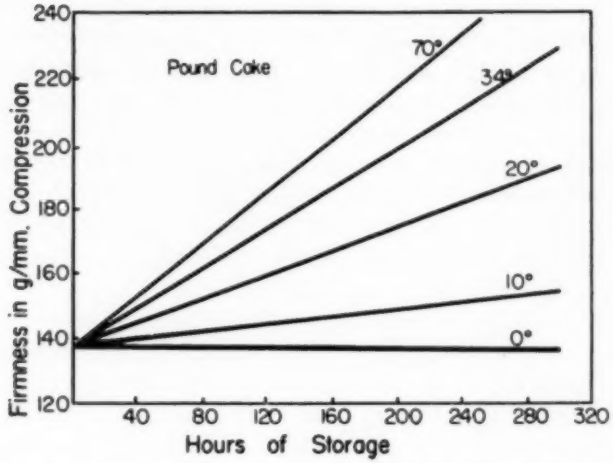

Fig. 4. Regression lines representing average rates of firming of pound cakes at various temperatures (°F.). Note difference in degrees of crumb compression used for this cake as compared to those in Figs. 1–3. Centigrade equivalents for the Fahrenheit temperatures shown are: 21°, 1°, −7°, −12°, and 18°C., respectively.

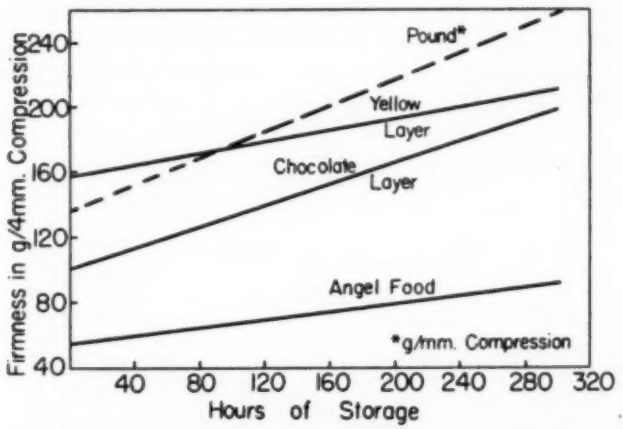

Fig. 5. Comparison of rates of firming of angel food and chocolate and yellow layer cakes at 70°F. (21°C.). Values for pound cake cannot be compared directly because of the different crumb compression used.

experiments was a wide fluctuation in firmness, among cakes of any one kind stored at a given temperature, that must have come principally from nonuniformity of the starting material. The analysis of variance of regression coefficients for the replicate runs indicated that only the three sets of chocolate cakes were from the same statistical population. All the other types showed a significant variation

($P = 0.05$) between sets. A principal reason why so few of the regression lines in Figs. 1 to 4 differed significantly among themselves probably is the high degree of this erratic fluctuation. A high degree of variability among cake samples was also found by Noznick and Geddes (7).

A measure of the variation among samples attributable, at least in part, to variation in the starting material is shown in Table I, which lists the variances (mean squares) of the linear components of the regressions (calculated from pooled results for the replicate storage experiments) along with the variances due to deviations from the regressions. The deviations are greatest for the yellow layer cake and

TABLE I

VARIANCES FOR LINEAR COMPONENTS AND DEVIATIONS FROM REGRESSION OF CAKE FIRMNESS ON STORAGE TIME

STORAGE TEMPERATURE	VARIANCE FOR LINEAR COMPONENT OF REGRESSION	VARIANCE FOR DEVIATION FROM LINEAR REGRESSION	F RATIO
°F.			
	Yellow cake		
70	75.26	8.23	9.15*
34	17.87	6.46	2.77
20	60.56	8.31	7.29*
10	34.75	6.62	5.25*
0	31.08	8.54	3.64
	Chocolate cake		
70	52.70	6.39	8.24*
34	42.79	1.73	24.74**
20	12.47	1.94	6.43*
10	11.49	1.76	6.52*
0	2.16	1.02	2.11
	Angel food cake		
70	11.03	0.46	24.24**
34	14.31	0.29	49.23**
20	4.73	0.30	15.88**
10	0.82	0.32	2.58
0	0.02	0.30	0.07
	Pound cake		
70	70.35	9.05	7.77*
34	53.47	7.36	7.26*
20	9.70	7.71	1.26
10	1.94	4.15	0.47
0	0.69	4.82	0.14

pound cake and least for the angel food and chocolate layer cake. There is little indication that the deviations are temperature-dependent except for the pound cake, which showed a tendency for progressively greater deviation variances as storage temperature increased.

This may be due chiefly to the higher firmness readings at the higher storage temperatures – that is, greater absolute differences but approximately the same relative differences among the firmness readings for the different samples. The low F ratios at the lower temperatures appear to result from such a slow rate of increase in firmness that the regression is nonsignificant in the face of the erratic variation among sample readings.

A number of variables during the production of these cakes could contribute to nonuniformity of a series of cakes, but two very likely ones are flour and eggs, especially since the cakes used in these experiments were baked at times several weeks apart. Other sources of variability, of course, enter during the progress of the storage experiment itself. Of these, effects due to different operators are most likely to be of serious consequence. Despite efforts to maintain uniformity, the cutting of cake sections for firmness measurements and the actual firmness measurements themselves are probable sources of significant variation (7). Their effect in the present experiments was demonstrated by arranging the final storage experiment so as to include a Latin-square test of the variables, one of which was operators. Four different operators cut up their own test pieces of cake and measured firmness. The variability thus assignable to operators was statistically significant ($P = 0.01$). Despite this, the expected variations due to main effects (cake types, storage temperatures, and storage times) were all significant at $P = 0.01$ when the variance due to operators was included in the error term used to test for significance.

In spite of the relatively large variability in results due to factors other than the effect of temperature on firming rates, the principal conclusions from the storage experiments are reliable and constitute successful attainment of the original objectives of the experiment. Moreover, the storage experiments, as conducted, provide a good example of the degree of variability encountered in typical retail bakery cakes of several kinds. Though necessarily very generalized as a result of the variability in test material in these particular experiments, the conclusions are thereby even more applicable to cakes in general.

Implications with Regard to Freezing Practices. The observation that cakes become firm more slowly as their temperature is lowered toward their freezing point explains why they are so tolerant to commercial freezing practices. In many operations, cakes are cooled only to 8° to 12°F. (−13° to −11°C.), so that they never actually become frozen. Yet these cakes remain in good condition for appreciable periods. This does not mean, however, that cakes need not be frozen for best results. The firmness curves at 10°F., for example, do not rise very

rapidly; but they do rise, so that within 1 to 2 weeks a detectable loss of softness will have occurred. Best results, therefore, require that cakes be held at 0°F. (−18°C.) or lower, if storage is to be prolonged more than a week or so. This recommendation agrees with those of others made on the basis of commercial experience (4, 5). The rather slow changes in cake firmness at reduced temperatures suggest that flavor or other changes may be the limiting factor in the storage life of frozen cakes.

The effect of temperature on rate of firming in cakes contrasts sharply with the opposite behavior of bread crumb (8) and the speed of retrogradation of starch (1, 2, 9), and suggests a different mechanism for the observed changes. Undoubtedly the large amounts of sugar, shortening, and eggs in most cakes are responsible in some manner for this difference in firming behavior. This inversion of behavior is of interest not only in itself but because of the practical importance with respect to the freezing of products intermediate in composition between bread and cakes. Those that behave more like bread at refrigerated temperatures should be frozen rapidly for best retention of softness, whereas those that are more like cake may be frozen more leisurely and more economically. Research is now in progress at this laboratory to determine which ingredients may be most important in modifying the firming behavior of baked goods and what levels are required to cause the behavior of a product to become like that of cakes at refrigerated temperatures.

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STUDIES ON WHEAT PLANTS USING CARBON-14 COMPOUNDS

VI. Some Observations on Protein Biosynthesis[1]

E. BILINSKI[2] AND W. B. MCCONNELL

ABSTRACT

Gliadin was the most radioactive of flour proteins isolated from wheat plants, the stems of which had been injected at various stages of maturity with either acetate-1-C^{14} or -2-C^{14}. Similar observations were made for glutamic acid isolated from the proteins, the results suggesting that gliadin reaches a maximum rate of biosynthesis at a later period than do glutenin, albumin, and globulin. Glutamic acid incorporated more methyl- than carboxyl-carbon from acetate, most differentiation between the two carbons being shown by gliadin. The likelihood that the glutamic acid of gliadin was derived from α-ketoglutaric acid that had undergone little recycling in the tricarboxylic acid cycle supports the idea that gliadin is an independently formed protein.

Bran proteins showed markedly increasing activity with late injection, suggesting their formation at still later stages of maturation.

The results are considered as an additional characterization of kernel proteins. The distinctive labeling pattern found for the protein fractions strongly suggests that each possesses a biosynthetic individuality rather than representing an arbitrary fractionation of a somewhat continuous spectrum of molecules as may be conceived of for the gluten complex.

By the injection of carbon-14 tracers into the stems of wheat plants, mature kernels are obtained in which carbon-14 is distributed nonuniformly among the kernel components (15). Acetate-C^{14} is particularly effective in labeling proteins, and this retention of substantial amounts of tracers from the time of injection until growth and maturation have ceased indicates that the storage of protein in the kernel is slowly reversible or irreversible. Certain amino acids are especially radioactive following administration of acetate-C^{14} and it has been possible to interpret data on the localization of carbon-14 within the molecules in terms of known biosynthetic pathways for amino acids (3, 4). These studies on amino acid and protein synthesis in plants find their parallel in animal studies done by Black and co-workers, who examined the incorporation of carbon-14 into casein by the lactating cow (6, 7). The close agreement between some results obtained by these latter workers and results of the present studies (4, 6, 7) are illustrative of the common occurrence of certain biosynthetic mechanisms in widely differing tissues.

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The distribution of tracers among kernel components depends in part upon the stage of development when the tracer was administered (14, 15). It appeared, therefore, that more detailed observation of the effects of time on localization of carbon-14 in the seeds could provide information on the sequence of some biochemical events during plant development.

This communication presents preliminary results of a study of the carbon-14 content of different kernel proteins from plants given acetate-1-C^{14} and -2-C^{14} at selected stages of maturation. The labeling of glutamic acid isolated from these protein fractions was also studied. Tentative suggestions regarding sequence of formation of the proteins are made.

Experimental Methods

Production of Labeled Wheat. Thatcher wheat was sown in a field plot on May 7, 1956. Good growing conditions were encountered throughout the season and no significant amount of damage by disease or insects was observed. The plants developed normally and the wheat was harvested fully mature on August 27 (114 days' growth) to yield plump kernels grading No. 1 Northern. On July 18 acetate-1-C^{14} was injected into the stems of each of twelve typical tillers (0.1 ml. containing 0.149 mg. sodium acetate and 10.0 μc. of carbon-14 per plant). Details of the method of administering tracer are described elsewhere (15). Similarly, twelve tillers were given acetate-2-C^{14} at the same dosage of sodium acetate and carbon-14. The injections were repeated on different tillers on July 21, 26, and August 3. At the time of the first injections the kernels were in "early dough" or late milk stage and at the last injections the kernels were well filled and firm and were slightly "mealy" when broken open. The heads were harvested at full maturity and air-dried at room temperature, and the kernels collected. Kernels from all twelve heads of a single experiment were pooled and 10 g. of each sample milled in an experimental mill to yield six fractions among which were flour (6.2 g.) and bran (1.6 g.).

Isolation of Proteins. Five grams of flour were dried under vacuum in the presence of phosphorus pentoxide and fractionated according to an arbitrary scheme (Fig. 1). Comments on the steps follow:

Ether extraction: Flour (5 g.) was extracted with dry ether for 15.5 hours in a Soxhlet apparatus. The residue was air-dried.

Salt Extraction: The "defatted" flour was mixed with 75 ml. of 0.1*N* sodium chloride at pH 7 and allowed to stand overnight at 4°C. (10). The mixture was then stirred for 15 minutes and the supernatant removed by centrifugation. The residue was stirred for 15 minutes with

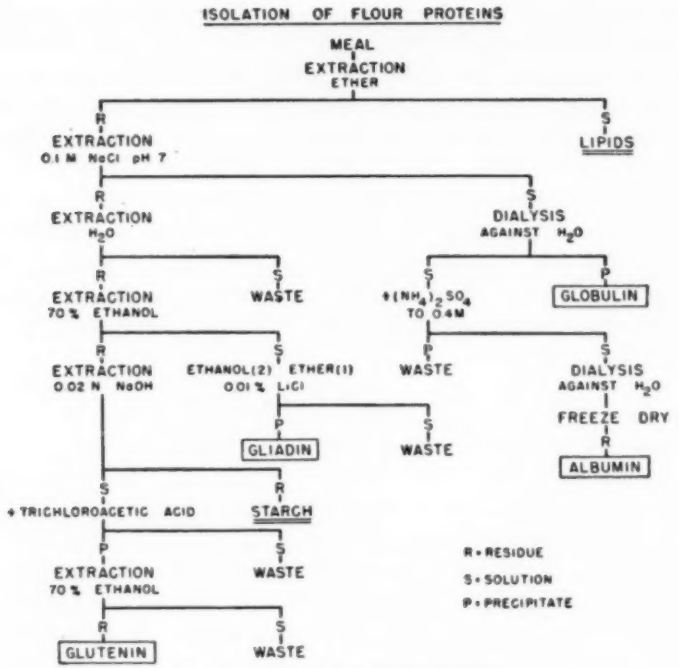

Fig. 1. Isolation of flour proteins.

another 50 ml. of salt solution and centrifuged. The supernatant solutions were combined for isolation of albumin and globulin and the residue used to prepare the gluten proteins.

Isolation of albumin and globulin: Sodium chloride and material of low molecular weight were removed from the above extract by dialysis at 5°C. against 4 liters of distilled water. Dialysis continued for 5 days, the water being changed every 12 hours. Protein precipitation accompanied the removal of salt. The precipitate was collected by centrifugation, washed with acetone and ether, and dried under vacuum (globulin yield 14 mg. per g. flour). Water was removed from the dialyzed solution by freeze-drying and the solid dissolved in 10 ml. of water.

Gliadin taken up in salt extraction was removed by addition of an equal volume of 0.8M ammonium sulfate (16). After standing for 1 hour at 5°C. a slight precipitate was centrifuged off and discarded. The supernatant was again dialyzed to remove salts and freeze-dried (albumin yield 16 mg. per g. flour).

Isolation of gliadin: The residue from salt extraction was freed of salt by washing four times with 25-ml. aliquots of distilled water. The

mixture was stirred for 10 minutes during each washing, and separation was achieved by centrifugation, the supernatant being discarded each time. An aliquot of the dried material (1.5 g.) was extracted for 2 hours at room temperature by stirring with 40 ml. of 70% ethanol (v/v). The supernatant was removed by centrifugation and the residue re-extracted three times with 10 ml. of 70% alcohol, with 10 minutes' stirring during each extraction. Gliadin was precipitated from the combined extracts by addition of 350 ml. of 0.01% lithium chloride (8) dissolved in a mixture of ethanol and ether (2:1). The precipitate was washed with acetone and ether and dried under vacuum (gliadin yield 45 mg. per g. of dried flour).

Isolation of glutenin: The residue from gliadin extraction was further extracted with 0.02*N* sodium hydroxide. Forty milliliters of alkali were used for the first extraction and 10 ml. for each of three extractions, the procedure being the same as used for gliadin preparation. Proteins soluble in the alkali were precipitated by stirring for 30 minutes with 50 ml. of 10% trichloroacetic acid. The precipitate was collected by centrifugation, washed with acetone and ether, and dried. Contaminating gliadin was removed by extracting the product overnight with 10 ml. of 70% ethanol and the residue dried under vacuum (glutenin yield 40 mg. per 1 g. of dried flour).

Three protein fractions were isolated from the bran. Because the quantities of starting material were limited to about 1.5 g., simplifications were introduced into the scheme used for flour. Albumin, which remained in solution after the original salt extraction and dialysis, was not further purified by precipitation with 0.4M ammonium sulfate. The residue of bran left following salt extraction was extracted with 0.02*N* sodium hydroxide and all protein collected in this manner was designated as "sodium hydroxide-soluble" protein. The approximate yields obtained per g. of bran were albumin 21 mg., globulin 20 mg., and sodium hydroxide-soluble protein 20 mg.

Isolation of Glutamic Acid. The proteins were hydrolyzed for 22 hours with 6*N* hydrochloric acid in sealed tubes immersed in boiling water. The hydrolysates were freed of hydrochloric acid by evaporation and humin removed by filtration of the aqueous solution. Glutamic acid was isolated from the hydrolysates by chromatography on Dowex-1, according to the method of Hirs, Moore, and Stein (12). Since many hydrolysates were to be chromatographed, the procedure was altered slightly to eliminate the use of a fraction collector and thus permit the simultaneous separation of a number of samples. A column of Dowex-1x8, 60 cm. by 2 cm., was used. Glutamic acid free of other amino acids was collected in the eluates (0.5*N* acetic acid) from 240 ml.

to 400 ml. Solvent was removed by evaporation at temperatures below 40°C., and after suitable additions of carrier the samples were counted.

Degradation of Glutamic Acid. The specific activity of carbon-1 of glutamic acid was determined from the specific activity of carbon dioxide liberated from glutamic acid by ninhydrin decarboxylation (19). Specific activity in carbon 5 was determined by counting carbon dioxide liberated by the Schmidt reaction (1, 17).

Determination of Specific Activity. Carbon-14 content of all samples was determined by conversion to carbon dioxide by wet combustion and counting the product as a gas (9).

Results and Discussion

Incorporation of Carbon-14 in Flour Proteins. The specific activity of proteins isolated from the flour clearly depends upon the stage of growth when the tracer was given (Fig. 2). The results, furthermore, show that there are marked differences between the specific activity of

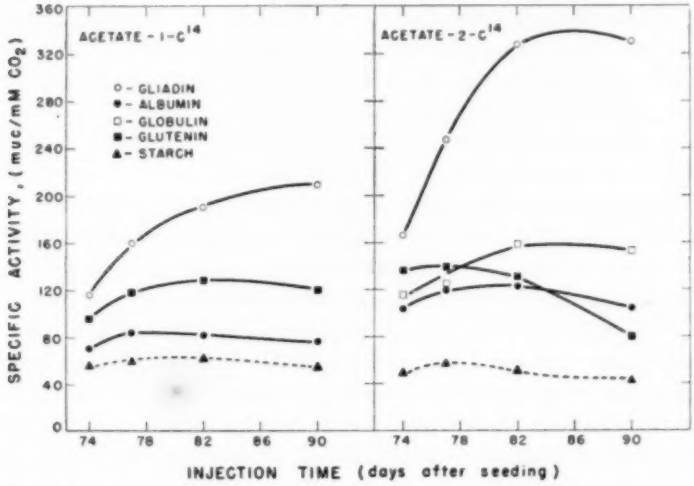

Fig. 2. Specific activity of flour proteins following administration of tracer at different stages of maturity.

different proteins isolated from any given sample of the wheat. A marked effect of time of injection on the specific activity of glutamic acid isolated from the proteins is also evident (Fig. 3), and notable differences between specific activities of glutamic acid from different proteins are observed. The proteins and the glutamic acid in particular are among the most active of flour components (compare with starch). Gliadin is the most active protein, glutenin next most active;

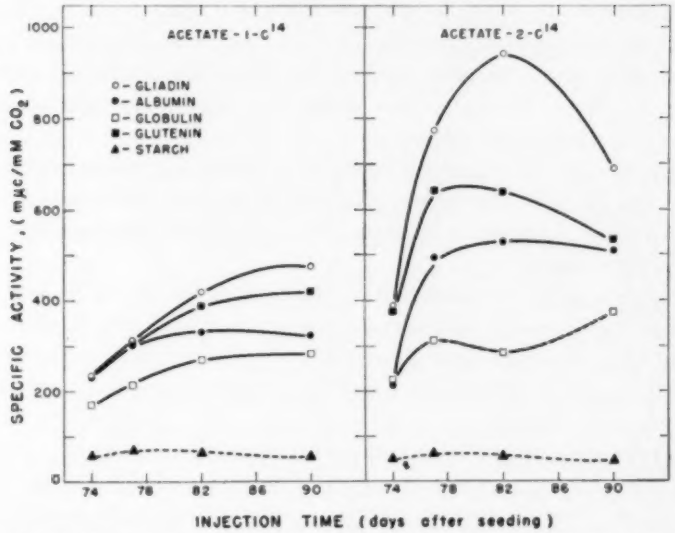

Fig. 3. Specific activity of glutamic acid from flour proteins with administration of tracer at different stages of maturity.

albumin and globulin are similar to each other in labeling and considerably less active than the gluten proteins. The specific activity of glutamic acid from gliadin, glutenin, and albumin are much the same

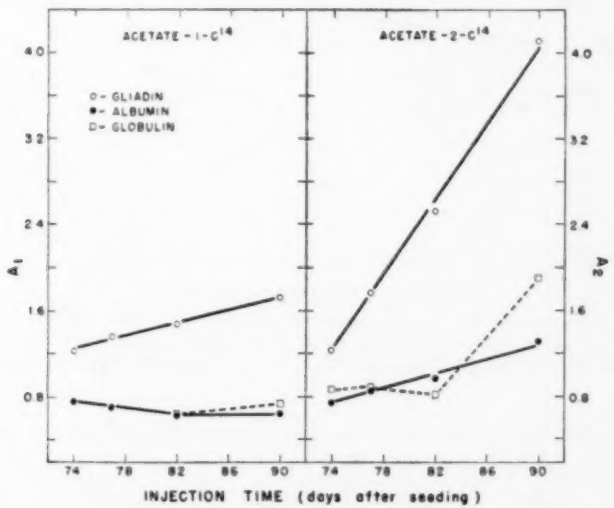

Fig. 4. Comparison of specific activity of flour proteins. A_1 and A_2 are the ratios of the specific activity of the proteins to the specific activity of glutenin from the same flour sample.

when acetate-1-C^{14} is fed at the two earliest stages, but differences are evident at later stages, the specific activity decreasing in the order: gliadin, glutenin, and albumin. Globulin yielded glutamic acid with lowest activity at all stages but unlike that from albumin a decrease with late injection did not occur.

With acetate-2-C^{14} gliadin again was clearly the most active at all stages, but as with the other proteins there was lower activity from late injection, maximum activity occurring following labeling at an intermediate stage.

Glutamic acid was consistently more active than the protein from which it was isolated and therefore contributes largely to the trends evident in protein labeling. The different glutamic acid content of the proteins no doubt affects the parallelism between protein activity and glutamic acid activity.

To provide a more direct comparison between the specific activities of the proteins the results are expressed in terms of the specific activity of glutenin (Fig. 4). Specific activities of glutamic acid are expressed in terms of the activity of glutamic acid from glutenin (Fig. 5). Glu-

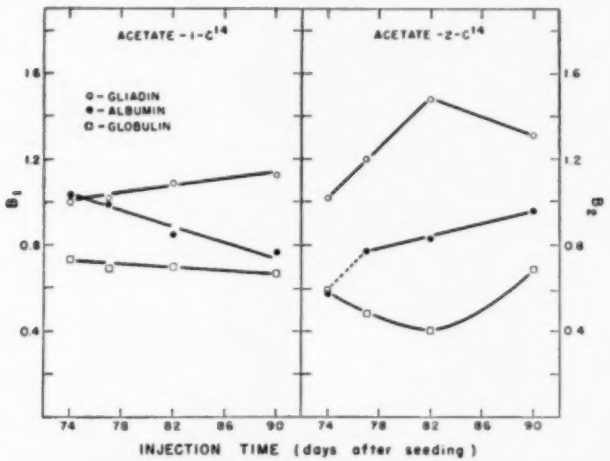

Fig. 5. Comparison of specific activities of glutamic acid samples. B_1 and B_2 are the ratios of the specific activities of glutamic acid isolated from the proteins to the specific activity of glutamic acid isolated from the glutenin of the same flour sample.

tenin was chosen as the basis for comparison because of its intermediate position in relation to other proteins (Fig. 3). In addition, the procedure tends to minimize variations between samples that may have been caused by technical factors such as mechanical loss of tracer or slight handling damage to the plants.

The specific activity of gliadin increases almost linearly with time relative to glutenin following administration of either acetate-1-C^{14} or -2-C^{14}. The appearance of comparatively large amounts of carbon-14 in gliadin with late injection indicates that as maturity is approached the emphasis in the wheat plant is upon gliadin deposition rather than upon glutenin deposition. This result is in accord with that of McCalla (13), who concluded from studies of wheat protein at different stages of maturity that the wheat protein fraction corresponding to gliadin is formed later in the life of the plant than the glutenin. Sharp and Elmer also reported increasing gliadin content during maturation (18). Other workers have obtained evidence for sequential formation of seed proteins. Danielsson (11), for example, found that the globulins of pea seeds increased rapidly at an early stage of maturation, but that the relative percentage of vicilin decreased while that of legumin increased as ripening proceeded. Bishop (5), in studying barley protein formation, found that although all major components studied were present throughout the ripening process their proportions changed, hordein increasing in proportion to total nitrogen with ripening and glutenin remaining almost constant.

The sequence of deposition for proteins other than gliadin is not clearly evident from present results. When acetate-1-C^{14} was used the albumin and globulin were less active than the glutenin, there being an indication that albumin decreased slightly relative to glutenin at

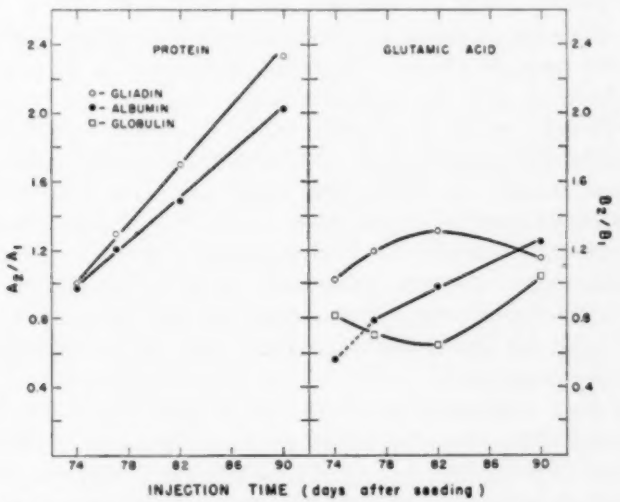

Fig. 6. Comparison of the utilization of carboxyl and methyl carbons of acetate for protein synthesis. A_1, A_2, B_1 and B_2 are as in Fig. 4 and 5.

later stages. With acetate-2-C^{14} the albumin was less active than glutenin when tracer was injected early, but its relative activity increased with time of injection until at the last stage it was considerably more active than glutenin.

Specific activity of glutamic acid from gliadin (Fig. 5) was as high as, or higher than, the specific activity of glutamic acid from corresponding samples of other proteins, its excess activity being more striking with later injection. This is, in general, the effect observed in the proteins, but with somewhat smaller differences. Comparison of the glutamic acid from albumin and globulin also shows the same general trends as observed with the proteins.

Differences in the relative incorporation of the carboxyl and methyl carbons of acetate into different proteins or glutamic acid samples are evident from Figs. 2 and 3 and Figs. 4 and 5. These differences may be demonstrated more readily by comparing the manner in which the ratios of specific activities of the proteins to glutenin at different times vary according to whether acetate-1-C^{14} or -2-C^{14} was administered (Fig. 6). The ordinate has the following mathematical significance:

$$\frac{\text{Sp. act. protein acetate-2-}C^{14}}{\text{Sp. act. glutenin acetate-2-}C^{14}} \Big/ \frac{\text{Sp. act. protein acetate-1-}C^{14}}{\text{Sp. act. glutenin acetate-1-}C^{14}}$$

A similar procedure was used for comparing the two tracers in labeling of glutamic acid. The ratios of specific activity from acetate-2-C^{14} to activity from acetate-1-C^{14} are in general greater than one indicating that the methyl group is incorporated to a greater extent than the carboxyl group (see Table II). When the tracers are given 74 days after seeding, the proteins albumin, globulin, and glutenin all tend to distinguish between the two acetate carbons to about the same extent (about 1.42 times as much carbon-14 from acetate-2-C^{14} as from -1-C^{14}). Difference in the proteins with regard to their incorporation of the two carbons became evident at later stages and an almost linear relation in these changes is indicated in Fig. 6, the proteins gliadin and albumin showing much more incorporation of methyl carbons. The observations made with the proteins themselves are repeated qualitatively in the results with glutamic acid, the differences, however, not being as large. An apparently anomalous behavior for globulin is reflected in a curved line.

It has been shown that the tricarboxylic acid (T.C.A.) cycle plays an important role in the biosynthesis of the dicarboxylic acids of wheat proteins (4). The localization of the carboxyl and methyl carbons of acetate in glutamic acid are thus quite different, carboxyl carbon tending to appear externally and methyl carbon internally. The terminal

carbons of α-ketoglutaric acid (immediate precursor of glutamic acid) are more readily lost with recycling in the T.C.A. cycle. It appeared, therefore, that differences in metabolic turnover may account for differences in the effectiveness of the two forms of acetate-C^{14} for labeling glutamic acid. The higher activities obtained with acetate-2-C^{14} would be in accord with this idea. Since the localization of tracer within the glutamic acid is particularly important for interpreting results observed, the specific activity of carbons 1 and 5 of glutamic acid from proteins labeled with acetate-1-C^{14} was determined (Table I).

TABLE I

LOCALIZATION OF CARBON-14 IN GLUTAMIC ACID OF PROTEINS AFTER INJECTION OF ACETATE-1-C^{14}

Source of Glutamic Acid	Injection Time (After Seeding)	Specific Activity (mμc/mM CO_2)			Glutamic Activity	
		Glutamic Acid	1-C	5-C	1-C	5-C
	days				%	%
Globulin	74	169	256	449	30	53
	77	211	316	543	30	52
	82	267	394	705	30	53
	90	280	404	737	29	53
Albumin	74	238	354	623	30	52
	77	301	426	787	28	52
	82	327	449	913	28	56
	90	321	427	858	27	54
Glutenin	74	231	322	633	28	55
	77	304	432	867	28	57
	82	384	510	1113	27	58
	90	418	546	1258	26	60
Gliadin	74	231	338	666	29	58
	77	307	422	925	28	60
	82	415	562	1260	27	61
	90	471	580	1460	25	62

If glutamic acid is formed by the amination of α-ketoglutaric acid, its labeling pattern may be deduced from that of the α-ketoglutaric acid. When α-ketoglutaric acid is formed by the T.C.A. cycle, direct incorporation of acetate-1-C^{14} on the first turn of the cycle yields glutamic acid labeled only in carbon 5. If the α-ketoglutaric acid recycles once, one-half of the carbon-14 appears as carbon dioxide and the remainder in position 1 of glutamic acid (2). Another recycling liberates the remainder of the carbon-14 as carbon dioxide. Recycling thus results in a rapid conversion of acetate carboxyl to carbon dioxide, and tends to increase the amount of acetate carboxyl appearing in carbon 1 of glutamic acid at the expense of carbon 5. In recycling, however, if oxaloacetate condenses with acetate-1-C^{14} of the same activity as in the first cycle, a steady state is reached after the second cycle with the specific activity of carbons 5 and 1 being in the proportions of 2:1 (2,

6). The carboxyl group of acetate cannot enter the interior carbons of acetate by the scheme considered above. The results of Table I indicate that 50–60% of the activity of glutamic acid is in position 5 and 25–30% is in carbon 1. The results are thus in accord with predictions from the T.C.A. cycle provided some recycling occurs, and it is probable that a condition approaching the steady state existed during biosynthesis of a number of the samples of glutamic acid. If, as is reasonable to assume, higher proportion of activity in carbon 5 to that in carbon 1 indicates less recycling, some conclusions can be drawn about the turnover of glutamic acid incorporated into the flour proteins. The ratios plotted in Fig. 7 suggest that for all proteins less recycling

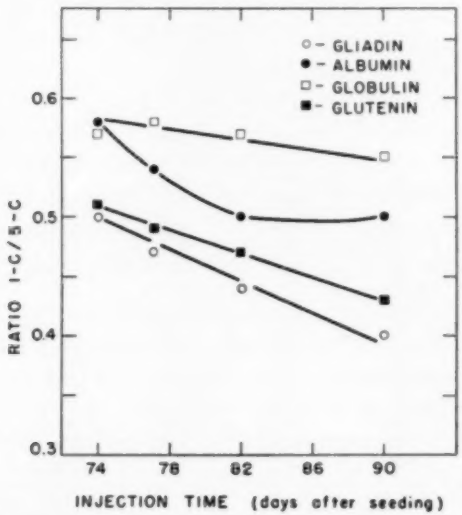

Fig. 7. Comparison of labeling in carbons 1 and 5 of glutamic acid from flour proteins. Acetate-1-C^{14} was used to label the kernels.

occurs when tracer is given later in the life of the plant. This is as would be expected. Figure 7 also indicates that glutamic acid of gliadin has recycled less than that from any other protein at a corresponding time of injection. Glutenin, albumin, and globulin appear to show increased recycling in the order named.

It was noted above that carboxyl carbon of acetate is readily converted to carbon dioxide by recycling twice, while methyl carbon appears first in the internal carbons and is more slowly oxidized. Nevertheless, although none of the methyl carbon of acetate is converted to carbon dioxide in the first two cycles, one-quarter of it becomes carbon dioxide on the third cycle and one-half of what remains is liberated

upon each additional cycle. It follows, therefore, that when recycling is extensive, both carbons of acetate are largely converted to carbon dioxide. The carbon dioxide would undoubtedly be partially reutilized for biosynthesis of kernel constituents, but there would be no differentiation between acetate-1-C^{14} and -2-C^{14}. Globulin, as an example, is less active than other proteins (Figs. 2 and 3) and tends to show less differentiation between the carbons of acetate. This is probably a result of more recycling in the T.C.A. cycle of the important amino acid precursor, α-ketoglutaric acid (Fig. 7).

Direct incorporation of acetate-C^{14} by way of the T.C.A. cycle without recycling would produce no carbon dioxide from either the carboxyl or methyl group of acetate and would not give rise to any differentiation between the groups. For this reason protein synthesized under these conditions would have exceptionally high activity. No protein isolated in the present experiment appears to fit this extreme picture. In further agreement with this is the observation that none of the proteins contain glutamic acid that has not been involved in at least some recycling (Table I).

It follows from the above discussion that maximum differentiation between the incorporation of methyl and carboxyl groups of acetate will be found at some intermediate amount of recycling. The amount of recycling giving the maximum difference between the two isotopes will depend upon the extent to which recycling oxaloacetate is condensing with radioactive acetate. It is probable that with the system studied, maximum differentiation corresponds to a small amount of recycling. The particularly large differentiation between methyl and carboxyl groups of acetate by glutamic acid in gliadin and the high activity (Table II) may thus reasonably be associated with fairly direct incorporation (little recycling). At the last stage of injection, activity of glutamic acid from gliadin is lower and the difference between in-

TABLE II

RATIO OF SPECIFIC ACTIVITIES FROM ACETATE-2-C^{14} TO CORRESPONDING SPECIFIC ACTIVITIES FROM ACETATE-1-C^{14}

MATERIAL	RATIO SPECIFIC ACTIVITIES FROM DIFFERENT STAGES OF INJECTION			
	74 Days	77 Days	82 Days	90 Days
Albumin	1.42	1.43	1.50	1.35
Globulin	...	...	...	...
Glutenin	1.42	1.18	1.02	0.66
Gliadin	1.42	1.54	1.72	1.58
Glutamic acid albumin	0.91	1.63	1.61	1.57
Glutamic acid globulin	1.30	1.46	1.05	1.33
Glutamic acid glutenin	1.61	2.10	1.64	1.26
Glutamic acid gliadin	1.65	2.50	2.26	1.45

corporation of the two tracers is less. The latter effect may be associated with less than "optimum" recycling and the lower activity, with lower over-all efficiency of acetate incorporation in the kernel, and with shift in emphasis from synthesis of flour protein to bran proteins (see Fig. 8).

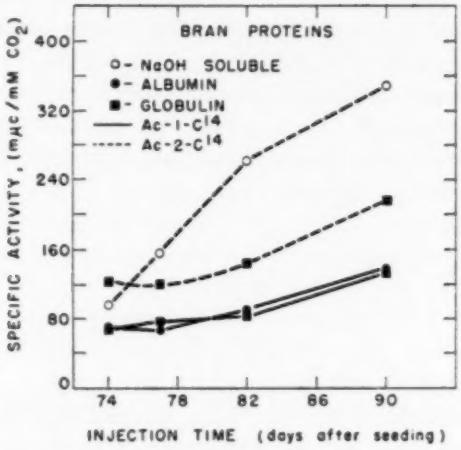

Fig. 8. Labeling of bran proteins.

The results show that different proteins isolated from a single sample of flour contain at maturity glutamic acid which has apparently been involved in different amounts of recycling. These differences probably reflect, in part at least, differences in the metabolic stability of the proteins. Gliadin thus appears to be formed irreversibly as compared with other proteins. Glutenin probably is involved in slightly more turnover, while albumin and globulin appear to be less stable than either of the gluten proteins (Fig. 7). No explanation is offered at the present time for the observation that albumin shows at some stages more differentiation between methyl and carboxyl carbon of acetate than does glutenin.

Since yields of protein and glutamic acid from bran were small, complete data were not obtained. The results (Figs. 8 and 9) are similar in several respects to those observed with the flour proteins. Acetate-2-C^{14}, for example, gave higher specific activities than did acetate-1-C^{14} for all corresponding samples of protein. The proteins were much more active than the bran as a whole and the glutamic acid, in turn, markedly more active than the proteins from which they were taken. In addition, the alkali-soluble proteins contained a higher concentration of tracer than did the water-soluble or salt-soluble proteins.

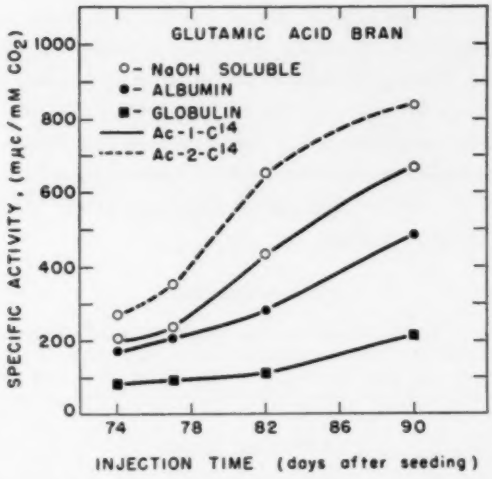

Fig. 9. Labeling of glutamic acid from bran proteins.

The effect of time on the amount of tracer incorporated is, however, quite different with bran proteins from that with the flour proteins. Late injection introduced most tracer into all the bran proteins and into the glutamic acid from them. The advantage of late injection for bran labeling is marked and in most cases a plot of specific activity against time of injection gave a curve that is concave upward. Results with flour proteins, if so plotted, would all give curves concave downward, and for many of them a maximum at an intermediate time would be noted.

It is concluded from the above observations that the bran proteins are more or less discrete components and are probably formed independently and at a later stage than the flour proteins, individual differences in bran proteins themselves being noted.

The over-all results of the present study with regard to the formation of the gluten proteins, gliadin and glutenin, are, as mentioned above, in accord with ideas put forth by McCalla (13). McCalla suggested that the potassium iodide-soluble material (gliadin) was the last formed, and concluded that the earliest-formed gluten fraction became the most insoluble portion of the protein in the mature kernels (glutenin). He also suggested that the nonprotein nitrogen in the kernel at any one stage of development is the precursor of a definite fraction of the endosperm protein, rather than of a portion of the whole gluten complex. Labeling observed in the present work not only indicates later biosynthesis of gliadin but suggests strongly that it has not been derived to any appreciable extent through interconversion of other

proteins present at an earlier stage. Gliadin at least appears to be formed independently of other proteins. Moreover, the results, by indicating some distinguishing features in the history of the kernel proteins of mature wheat, may be considered as an additional characterization of these proteins. They constitute an argument for regarding the fractions as individual protein types rather than as artificially separated parts of a more or less continuous spectrum of molecules constituting the gluten complex.

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A NOTE ON THE DEVELOPMENT OF THE STARCH OF THE RIPENING BARLEY EAR[1]

G. HARRIS AND I. C. MACWILLIAM

Recent studies on starches taken from wheat (2) and maize (6, 8) at different stages of ripening showed that there is an increase both in the average size of the granules and in the proportion of amylose in the starches as the ear develops. It has now been found that these changes are paralleled in barley.

Barley plants were collected from a selected portion of a field on June 24 and at weekly intervals thereafter, and the grains from a known number of ears of each sample were freed from awns, spikelets, and other contaminating materials. Enzymes present were inactivated by heating the grains under reflux for 3 hours in 80% ethanol, allowance being made for the water present in the added grain. The grains were filtered off, ground in a Wiley mill, and extracted exhaustively with 80% ethanol in a Soxhlet apparatus. Starch was then extracted from the ground residue by the method of Pucher, Leavenworth, and Vickery (7), using perchloric acid. The dissolved starch was precipitated with iodine to free it from contaminating polysaccharides, redissolved in dilute alkali, and estimated by means of the anthrone reagent using the method of MacWilliam, Hall, and Harris (4). The "blue values" of the starches were determined by the method of Bourne, Haworth, Macey, and Peat (3), that portion of the above alkaline solution calculated to contain 5 mg. of starch being acidified and treated with iodine under the stipulated conditions. The values obtained for the starches of two different barley varieties are listed in Table I. The relative amounts of polysaccharides in the starch samples were calculated (Table I), on the assumption that the blue values of barley amylopectin and amylose are 0.05 and 1.30 respectively (1, 5). The results indicate that during the early period of development of the grain (samples 1 to 7) when the major part of the starch is being synthesized, the percentage of amylose in the whole starch progressively increases. This is interpreted to mean that the rate of amylopectin synthesis relative to amylose formation is greater in the first few weeks than in the subsequent period. It will be observed that the samples from both varieties of barley studied show similar trends, although in the Carlsberg variety the rapid synthesis of starch commenced a little earlier (compare sample 3) than in the Spratt-Archer sample.

[1] Manuscript received June 24, 1957. Contribution from the Brewing Industry Research Foundation, Nutfield, Surrey, England.

TABLE I

CHANGES IN THE STARCH OF TWO VARIETIES OF BARLEY DURING RIPENING

SAMPLE NO. (SEE TEXT)	STARCH (PERCENT IN DRY GRAIN, W/W)		BLUE VALUE[3] OF STARCH		AMYLOSE CONTENT OF STARCH	
	Carlsberg	Spratt-Archer	Carlsberg	Spratt-Archer	Carlsberg	Spratt-Archer
					%	%
1	2.4	2.2	0.214	0.227	13.1	14.2
2	6.1	2.4	0.243	0.236	15.4	14.9
3	19.1	5.9	0.334	0.271	22.7	17.9
4	39.1	37.2	0.333	0.371	22.6	25.7
5	57.3	54.4	0.345	0.354	23.6	24.3
6	62.3	60.5	0.346	0.351	23.7	24.1
7	63.8	63.0	0.369	0.367	25.5	25.2
8	65.5	66.0	0.370	0.368	25.6	25.4
9	64.0	66.9	0.366	0.367	25.3	25.4
10	64.9	64.0	0.368	0.371	25.4	25.7
11	65.9	65.2	0.362	0.372	25.0	25.8
12	65.3	65.5	0.365	0.376	25.2	26.1

Attempts were made to separate the starch granules from the ground material of samples 1 to 3 (Table I) by the method of MacWilliam and Percival (5), but the material could not be freed from fiber and green-colored material. However, when the fibrous mass was stained with iodine and examined under the microscope, the stained granules were found to be considerably smaller than the mature granules found in samples 7 to 12 (Table I). The average granule size increased progressively in samples 4 to 6.

These results for barley starch taken in conjunction with those obtained earlier for wheat and corn starches indicate that the phenomena of increase (a) in average size of granule and (b) in the proportion of amylose are general features of starch formation in cereals.

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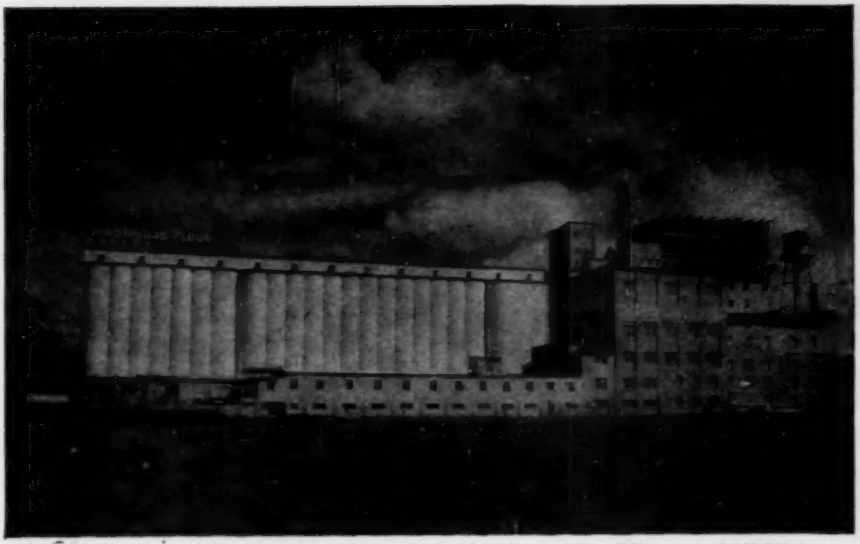

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